2020

Early Host Defense Mechanisms against La Crosse Virus Infection

Maria A. Cruz Zamora
University of Central Florida

EARLY HOST DEFENSE MECHANISMS AGAINST LA CROSSE VIRUS

by

MARIA A. CRUZ ZAMORA
M.S. University of Central Florida, 2016
B.S. University of Central Florida, 2013

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2020

Major Professor: Griffith Parks

ABSTRACT

Given the importance of innate defense mechanisms in the skin, we have examined the interactions of the arbovirus La Crosse virus (LACV) with serum factors that serve as a first line of antiviral defense, and the outcome of LACV infection of human keratinocytes, one of the main cell types present at viral entry. Incubation of LACV derived from insect cells (I-LACV) with normal human serum in vitro did not result in neutralization, but instead stabilized LACV virions and enhanced infectivity. Enhanced infectivity was also seen with heat inactivated serum devoid of complement activity and with serum from a range of animals including mouse, ferret and non-human primates. Depletion of antibodies from serum removed enhancement of I-LACV infectivity and sucrose gradient sedimentation assays showed IgG co-sedimenting with I-LACV particles. Serum-enhancement of LACV infectivity was not seen with virus derived from human cells, suggesting that insect cell-derived LACV is unique in its ability to subvert factors in serum to facilitate the initial infection of animal cells. In modeling initial replication following delivery of insect-derived virus to the skin, we show that I-LACV replication in HaCaT cells was restricted in culture by an antiviral response elicited by both IFN-β and IFN-λ. Media from I-LACV-infected cells induced killing of bystander non-infected HaCaT cells, and this cell death was relieved by blocking IFN-β signaling. Bystander cell killing was not seen with I-LACV infection of a human fibroblast cell line. Our data suggest that keratinocytes produce IFNs which limit virus spread through both antiviral signaling and by induction of cell death of potential new target cells for infection. These results are further evidence that virus and host immune interactions are complex and raises the question on how the combined outcome of these interactions determines the success of a virus infection and dissemination.

Dedicated to my parents Olga and Gustavo in honor of their sacrifices, struggles, and hard work to give me the opportunity to receive an education. Thank you for your unconditional love, for believing in me, and for all your guidance and support.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to Dr. Parks, for his support, patience, and guidance. It is immeasurable how much he has taught me and I am extremely grateful for having worked with him during these six years. I also want to thank my committee members; Drs. Khaled, Jewett and Rohde, their expertise and advice have been beyond valuable for this dissertation and for my professional development.

I want to thank current and previous members of the Parks lab, I greatly appreciate your intellectual input and the skills you have taught me. I'd like to acknowledge the help and effort of the support staff at the Burnett School of Biomedical Sciences for all their hard work in maintaining a positive work environment.

I want to thank my husband and my family for their love and encouragement, as well as my friends for all their help and support over these past six years.

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LIST OF ACRONYMS (or) ABBREVIATIONS

Abs -- Antibodies

ADE – Antibody- mediated enhancement

AP-1 – Activator protein-1

BSA – Bovine serum albumin

BUNV – Bunyamwera virus

C – Chicken

CNS – Central nervous system

DC-SIGN – Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

Dep – Depleted

DISC – Death-inducing signaling complex

DMEM – Dulbecco's modified Eagle medium

DMSO – Dimethyl sulfoxide

dsRNA – Double-stranded RNA

EBOV – Ebola virus

ER – Endoplasmic reticulum

F – Ferret

FADD – Fas-associated protein with death domain

FBS – Fetal bovine serum

G – Glycoprotein

H – Horse

HaCaT-LACV – HaCaT-derived LACV

HI – Heat Inactivated

HIV – Human immunodeficiency virus

Hpi – Hours post-infection

Hr – Hour

Hu – Human

I-LACV – Insect-derived LACV

IFIT1 – Interferon-induced protein with tetratricopeptide repeats 1

IFN – Interferon

IFNAR – Interferon alpha/beta receptor

IFNLR – Interferon lambda receptor 1

Ig – Immunoglobulin

IL – Interleukin

IRF – Interferon regulatory factor

ISG – Interferon stimulated genes

ISRE – Interferon stimulated response elements

IVIG – Intravenous immunoglobulins

JAK – Janus activated kinase

JNK – C-Jun NH2-terminal kinase

L – Large

LACV – La Crosse virus

M – Medium

MAVS – Mitochondrial antiviral signaling protein

MBL- Mannan-binding lectin

MOI – Multiplicity of Infection

mRNA – Messenger ribonucleic acid

MxA – Human myxovirus resistance protein 1

N – Nucleoprotein

nAbs – Natural antibodies

NF-κB – Nuclear Factor κB

NHS – Normal Human Serum

NK – Natural killer (cells)

NS – Non-Structural protein

OAS2 -- 2'-5'-oligoadenylate synthetase 2

P – Primate

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PFU – Plaque forming unit

pH – Potential for hydrogen

PI – Propidium Iodide

PKR – Protein kinase R

PRR – Pattern recognition receptors

qPCR – Real-time polymerase chain reaction

Rab – Ras-associated binding (proteins)

RIG-I – Retinoic acid inducible product-I

RNA – Ribonucleic Acid

RNP – Ribonucleoprotein complex

RPB1 – DNA-dependent RNA polymerase II subunit B1

RT-PCR – Reverse transcription polymerase chain reaction

Ruxo – Ruxolitinib

S – Small

SDS – Sodium dodecyl sulfate

STAT – Signal transducer and activator of transcription

TLR – Toll-like receptor

TNF – Tumor necrosis factor

UV – Ultraviolet (light)

CHAPTER 1: INTRODUCTION

La Crosse Virus Encephalitis

La Crosse virus (LACV) is a member of the *Peribunyaviridae* family within the order *Bunyavirales*. This order of virus is comprised of negative sense single stranded RNA viruses that are primarily transmitted through arthropod vectors, with the exception of the *Hantavirus* and *Arenavirus* families. Several members of the *Bunyavirales* order are significant human pathogens such as Rift Valley fever virus, Crimean Congo hemorrhagic fever virus, and Jamestown Canyon virus. There is a strong need for vaccines and therapies against these viruses as there are currently no approved therapeutic treatments (Albornoz et al., 2016).

LACV is primarily transmitted by the *Aedes triseratus* mosquito. Typically infection causes a mild febrile illness, however in a small subset of pediatric cases LACV infection leads to meningoencephalitis, seizures, and paralysis (Elliot, 1990; McJunkin et al., 2001). LACV is the leading cause of pediatric arboviral encephalitis in the USA, as of 2018 a total of 83 neuroinvasive cases were reported (Rust et al, 1999; ArboNET, CDC, 2019; Evans & Peterson, 2019). However, the actual number of LACV infection cases is estimated to be much higher, numbers are hard to calculate as there is underreporting of non-neurological cases due lack of distinct symptoms (Grimstad et al., 1984; Haddow & Odoi 2009). There are currently no approved therapeutic treatments for LACV infection and due to increase vector range and introduction of new potential vectors to endemic areas, LACV is considered and emerging threat in the eastern United States (Evans & Peterson, 2019; Gerhardt et al., 2001; Harris et al., 2015).

The structure of LACV has been well characterized. The genome of LACV is comprised of three negative sense single stranded RNA genomic segments: Small (S), Medium (M) and Large (L) segments, each of which is encapsidated by a nucleoprotein N encoded in the S segment. Attached to each genomic segment is a RNA dependent RNA polymerase subunit, encoded in the L segment. The segments are enclosed in a viral envelope derived from the host cell and anchored to the envelope are two glycoproteins the Gc and Gn, encoded by the M segment, which allow for attachment and internalization. In addition to the structural proteins, LACV encodes two non-structural proteins, one in the S segment, NSs, which has been shown to act as a virulence factor and IFN antagonist and one in the M segment, NSm, whose function is not well characterized but is thought to be involved in virion packaging (Borucki et al., 2002; Gonzalez-Scarano et al., 1988; Elliot, 1990).

La Crosse Virus Dual Life Cycle

Arboviruses such as LACV have a unique dual life cycle where they replicate both in arthropods and in mammalian hosts. Female mosquitoes acquire the virus when feeding from an infected host. For LACV, these hosts are generally small mammals such as squirrels or chipmunks which have been shown to be natural reservoirs for the virus. Once in a mosquito, the virus initially infects mosquito midgut cells, and then escapes the midgut and disseminates to the hemocoel, the heart neural ganglia, fat body, ovaries and finally to the salivary glands where it replicates to high titers (Borucki et al., 2002). LACV has been reported to infect the reproductive system in mosquitoes leading to vertical transmission to eggs which ensures LACV survival during winter months (Watts et al., 1973). Horizontal (venereal) transmission between infected

mosquitoes and uninfected mosquitoes has been reported as well (Thompson & Beaty, 1977, 1978). Together these methods of transmission remove the need of a mammalian host for LACV maintenance which suggests that arboviruses might have first evolved as insect only viruses (Marklewitz et al., 2013). Mosquitoes carrying LACV show few adverse signs of infection, nevertheless the infection is not entirely asymptomatic. There are reports that infected female mosquitoes have changes in their feeding behavior and mate more efficiently than their uninfected counterparts, suggesting that LACV affects mosquito behavior to ensure its spread to mammalian hosts (Jackson et al., 2012; Reese et al., 2009).

Infected female mosquitoes deposit the virus upon feeding on a mammalian host. Generally these hosts are mammals endemic to the same areas as the mosquitoes. Humans are considered accidental dead-end host where exposure occurs by chance. Once in the host, the virus replicates in local tissues and eventually disseminates to striated muscle where extensive replication occurs, resulting in a high viremia. After a peripheral viremia, the virus crosses the blood brain barrier through an unknown route and targets neurons leading to infection of the CNS (Johnson R., 1983; Bennett et al., 2008). The ability of LACV to disseminate into the brain is age-dependent, where young animals are more susceptible to this neural invasion. However, studies have shown that if peripheral infection and dissemination is bypassed and the virus is directly introduced into the brain, the same neurovirulence is observed across age groups. This suggests that peripheral virus replication determines virus pathogenesis (Bennett et al., 2008).

It is well established that invertebrate and vertebrate cells can differ in many cellular functions and pathways which are key cell components used by viruses during replication, including post-translational machinery and cell membrane compositions (Peterson K.J et al.,

2004; Yap et al., 2017). Thus, interactions with host factors and pathways can differ substantially between viruses derived from insect cells and viruses derived from mammalian cells. This has been shown to be true with Dengue virus, which has differential patterns of glycoprotein glycosylation between virus derived from insect and mammalian cells (Hacker et al., 2009). Insect cell-derived West Nile virus and Sindbis virus have been shown to bind DC-SIGN, an attachment factor on dendritic cells, to higher levels than the respective mammalian virus (Klimstra et al., 2003). In addition, the insect cell-derived alphaviruses Ross River virus and Venezuelan equine encephalitis virus have been shown to be poor inducers of IFN in contrast to the respective mammalian-derived counterparts (Shabman et al., 2007).

La Crosse Virus Cell Cycle

LACV entry into a host cell involves attachment of the glycoproteins, specifically Gc for mammalian cells, to cell receptors. The cell receptors of LACV are not known although studies show that DC-SIGN in dendritic cells could be a potential receptor, though not the only one as LACV infects a wide range of tissues and cell types (Hoffman et al., 2013). Entry into the cells after virus-cell attachment occurs via clathrin-mediated endocytosis. Hollidge et al. (2012) have shown that LACV entry is dependent on cellular proteins Rab5 but not Rab7, indicating that trafficking into early but not late endosomes is required for infection.

In the endosome the Gc protein undergoes a pH-dependent conformational change and triggers fusion of the viral envelope and the endosomal membrane (Plassmeyer et al., 2005, 2007). After this fusion event, the RNA genome encapsidated by nucleoprotein is released into the cytoplasm where primary transcription occurs. Primary transcription involves one mRNA

being transcribed from each of the three segments by the RNA-dependent RNA polymerase (L protein). Primary transcription requires the use of mRNA primers that are taken from cellular mRNA through a methylated cap-dependent endonuclease on the L protein. These primer sequences are made up of 12-18 nucleotides derived from the 5' end of cellular mRNAs, and the sequences of these caps are heterogenous, although preferences for specific motifs have been reported (Patterson et al., 1984). Studies have shown that LACV transcription requires simultaneous ongoing translation by host ribosomes (Barr, 2007; Kolskofsky et al., 1987). After primary transcription, polymerase activity switches to genome replication; the basis for this switch to genome replication is unclear, although reports suggest that the N protein may play a role (Eifan & Elliot, 2009; Walter et al., 2011). Genome replication requires the synthesis of an anti-genome which is produced in a primer independent manner from the full negative sense genome. Both the antigenome and nascent genome are immediately encapsidated by the nucleoprotein to prevent the formation of dsRNA, a pathogen-associated molecular pattern that can elicit immune responses in the cell (Walter &Barr, 2011; Elliott, 2014). Viral assembly and packaging have been extensively studied in a prototype bunyavirus, Bunyamwera Virus (BUNV), and it is thought to be very similar to LACV assembly. In BUNV, viral assembly occurs in the Golgi apparatus where insertion of the glycoproteins modifies the Golgi and allows the formation of tube-like viral factories that are connected to the rough ER and the mitochondria (Matsuoka et al., 1991; Novoa, Calderita, Cabezas et al., 2005; Fontana et al., 2008; Salanueva et al., 2003). The factories allow viral proteins specifically RNPs and glycoproteins to accumulate and interact. Packaging of viral proteins and nascent genome as well as envelope formation occur in the swollen lumen of the Golgi, resulting in an immature viral intermediate type I

(Salanueva et al., 2003). The first maturation step occurs in the trans-Golgi membranes where glycoproteins are N-glycosylated resulting in a partially infectious immature intermediate type II. Final maturation occurs during virus egress through the exocytic cell pathway when mature glycoprotein spikes are built and the virus becomes fully infections and is released via fusion of exocytic vesicles with the plasma membrane (Novoa, Calderita, Arranz, et al., 2005; Sanz-Sanchez & Risco 2013).

Host Defense Mechanisms at the Skin

Innate host immune mechanisms are the primary line of defense against infection by viruses. These non-specific mechanisms can included mechanical barriers such as skin and mucosa, antiviral inhibitors such as opsonins and antimicrobial peptides, and soluble factors such as IFN and cytokines (Koyama et al., 2008; Barber, 2000).

Mechanical Barriers

The skin is a complex organ that plays a vital role in protection against external pathogens. It is composed of an elaborate structure that includes the outermost epidermis and an inner dermis. The epidermis is mainly composed of layers of differentiated keratinocytes. The outermost layer are terminally differentiated corneocytes that help prevent the entry of microbes as well as dehydration of underlying layers. It also provides a mechanical protection against abrasion and subsequent pathogen entry for the more fragile layers. The innermost layer consists of living keratinocytes that form a living layer due to tight junctions between the cells (Briant et al., 2014). Keratinocytes have a key innate role in the defense against pathogens since they

express basal or inducible levels of many pattern recognition receptors such as RIG-I and Toll-like receptors (TLRs) that can recognize a wide variety of pathogens (Kalali et al., 2008; Lebre et al., 2007; Pivarscsi et al., 2003). They can also express a range of immunomodulatory cytokines including interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor family proteins (TNFs), and IFNs in response to pathogen exposure (Fujisawa et al., 1997; Grewe et al., 1995; Howie et al 1996; Kock et al., 1990; Kupper et al., 1986; Larsen et al., 1989; Oxholm et al., 1991). The epidermis also hosts a number of Langerhans cells which are a dendritic cell-like resident immune cell that can capture pathogens and migrate to lymph nodes to activate other effector cells (Kubo et al., 2009). Below the epidermis, the dermis is composed of fibroblasts whose main function is to secrete proteins that form the extracellular matrix. These cells can also respond to pathogen exposure by producing Interferon type I. In addition to fibroblasts, this layer of the skin also contains many immune cells such as mast cells, macrophages and neutrophils. It is also interspersed with draining lymphatics and blood vessels that allow efficient recruitment of immune cells upon pathogen entry (Heath & Carbone, 2013; Briant et al., 2014).

Together the skin comprises of both an inert mechanical barrier and an active immune organ that work together to limit exposure and control replication of invading pathogens.

Serum Factors

For pathogens that enter through a blood meal such as arboviruses, it is likely that the interaction between arbovirus and blood represents the first interface between an arbovirus and innate immune factors. Factors in blood, specifically in serum, can have broad antiviral activities and are comprised of several molecule classes such as lipoproteins, soluble proteins,

glycoproteins, and polysaccharides (Singh et al., 1999; Baron et al., 2000). They have been shown to act by preventing virus attachment to the cell, by direct virus neutralization of viruses or by inhibiting virus replication (Blue et al., 2003; Gobet et al., 1988; Ochsenbein et al., 1999). Two of the most well-characterized antiviral factors in serum are complement and natural antibodies.

Complement is an ancient defense mechanism that evolved almost 700 million years ago against pathogens. The complement system is comprised of a series of proteins that upon pathogen recognition, undergo proteolytic cleavage in a cascade. As a result of this proteolytic cascade, pathogens can be directly lysed due to the formation of a membrane attack complex on the pathogen surface or opsonized by proteolytic fragments for enhanced uptake by phagocytes (Blue et al., 2003; Stoermer & Morrison, 2011). In addition, some complement fragments have been shown to act as soluble anaphylatoxins which help to recruit immunocompetent cells and initiate inflammatory responses (Blue et al., 2003). The role of complement in virus pathogenesis is complex, with some studies suggesting that complement has a protective role against virus infection. For example, West Nile Virus and Dengue Virus are neutralized by mannose binding lectin (MBL)-mediated activation of complement pathways, and mouse studies have shown complement-mediated clearance of Dengue virus from the host (Fuchs et al., 2010; Avirutnan et al., 2011). Other studies however suggest that complement can enhance disease severity, as seen with Dengue virus, where complement activation has been linked to enhanced severity in Dengue hemorrhagic shock (Bokisch et al., 1973; Nascimento et al., 2009).

Antibodies are generally considered part of the adaptive arm of the immune system. However, natural antibodies (nAbs) are polyreactive antibodies present in serum before exposure

to any pathogens and thus, are considered part of innate immunity. Natural antibodies can recognize a wide range of molecules including phospholipids, glycoproteins and glycolipids. They are known for their broad activity against self-antigens generated in normal cell processes such as oxidation and apoptosis, however they also provide protection against viral, bacterial, fungal and protozoan pathogens (Boes et al., 1998; Holodick et al., 2017; Ochsenbein et al., 1999; Subramaniam et al., 2010). In human, they are comprised mainly of IgM, IgG, and IgA classess. IgM nAbs are the most abundant and have been shown to have activity against various viruses such as influenza, vesicular stomatitis virus and lymphocytic choriomeningitis virus (Holodick et al., 2017; Palma et al., 2018; Baumgarth et al., 1999) (Gobet et al., 1988). Through the formation of a complex with soluble host lectins, IgGs have also been shown to recognize pathogens and to enhance phagocytosis by macrophages (Panda, Zhang, Yang et al., 2014; Panda, Zhang, Tan et al., 2013). Natural antibodies have been shown to be vital in protection against infections; this is further supported by the therapeutic use of polyclonal immunoglobulins - intravenous immunoglobulin (IVIG) - that contain nAbs and are commonly used for antibody replacement therapy in primary and secondary immunodeficiency patients (Kaveri et al., 2011).

Interferon Response

The most well characterized cell-derived response to viruses is the interferon (IFN) system. IFNs are a key antiviral group of cytokines that can have multiple effects on cells. In response to virus infection, they can induce an antiviral state in nearby uninfected cells to prevent virus spread, they can inhibit cell growth, and can promote apoptosis (Barber, 200; Biron & Sen, 2007). IFNs can also activate immune cells such as NK cells and macrophages and they

can induce B cells to switch immunoglobulin type, and alter T helper response (Biron et al., 1999; Le bon et al., 2001). There are three distinct classes of IFNs: Interferon type I, type II, and type III. Interferon type II is restricted to immune cells and is outside the scope of this thesis work. The most well characterized interferons are Type I interferons, primarily IFN-α and IFN-β. Type I IFNs can be produced by most nucleated cells, and most cells also have the capacity to respond to them (Sen, 2001). Recently discovered type III IFNs, IFN Lambda (λ)1, λ2, and λ3, have restricted expression that is limited to tissues of epithelial origins such as the skin (Zhou et al., 2018). Type I and type III interferons have been shown to act through the same pathways although their cognate receptors are different (Lazear et al., 2019). The receptor for type I IFN is the IFNAR receptor and it is ubiquitously expressed in most cells. The receptor for Type III is the IFNLR receptor which shares homology with IL-28 receptor (Lazear et al., 2019). Induction of both type I and type III IFN can occur in response to detection of viral RNA or other viral byproducts recognized by pattern recognition receptors (PRRs) in cells. These PRRs then signal through adaptor proteins such as mitochondrial antiviral-signaling protein (MAVS) or Myd88 to activate transcription factors including IFN regulatory factors (IRFs) and nuclear factor kappa B (NF-κB). Transcription factors IRF-3, NF-κB, and AP-1 translocate to the nucleus and activate transcription and translation of type I and/or type III IFNs. Following IFN production, newly secreted IFN can bind to its cognate IFN receptor in a paracrine or autocrine manner and initiate signaling. Bound receptors bind to Janus kinases leading to phosphorylation of the kinases and recruitment of STATs generally STAT1 and STAT2. Recruited STATs are phosphorylated, which allows them dimerize (heterodimers composed of STAT1 and STAT2) and associate with IRF9 to form a complex that then translocates to the nucleus where it binds to IFN stimulated

response elements (ISRE) and activates transcription of many IFN-stimulated genes that have antiviral properties (reviewed by Stark et al., 1998).

These IFN stimulated genes (ISGs) can function in multiple ways to restrict virus replication and spread. For example, 2'-5'-oligoadenylate synthetase 2 (OAS2) catalyzes the synthesis of 2',5'-oligoadenylates (2-5As). These molecules then activate latent RNase L which results in viral RNA degradation (Choi et al., 2015; Hovanessian & Wood, 1980). Another potent ISG is an antiviral RNA-binding protein IFIT1 that specifically binds single-stranded RNA bearing a 5'-triphosphate group (PPP-RNA), a specific characteristic of viral mRNA. Binding of IFIT1 to viral mRNA then results in inhibition of viral mRNA expression (Pichlmair et al., 2011). There are hundreds of ISGs that can potentially be expressed as a results of pathogen detection, many of whose functions are still not fully described.

Innate Immunity against LACV

Type I interferon pathways can play a role in protecting mice from lethal bunyavirus infections, acting in a potential range of cell types to limit dissemination or regulate neuroinvasion (Daniels et al., 2014; Taylor et al., 2014). In mice, myeloid dendritic cells (DC) are a key source of IFN in response to LACV that can control neurological disease, and is primarily driven by endosomal TLRs and RIG-I detection of viral RNA (Taylor et al., 2014). Other key components in the IFN response in non-myeloid cells include signaling through MAVS to activate IRF-3, IRF-5, and IRF-7 (Proenca-Modena et al., 2016). Type I IFN signaling can then induce expression of antiviral ISGs, including protein kinase R (PKR), IFN-induced protein 44 (IFI44), and viperin, which have been shown to inhibit replication of some

Bunyaviruses (Carlton-Smith & Elliot, 2012). In the case of LACV, the GTP binding protein MxA has been shown to prevent the accumulation of viral transcripts and proteins, possibly through trapping of viral nucleoprotein in perinuclear vesicles (Frese et al 1996; Georg et al., 2002; Landis et al., 1999). Prior work has shown that the LACV nonstructural protein NSs can prevent type I IFN induction in some cell types by degradation of the RBP1 subunit of RNA polymerase II (Verbruggen et al., 2011). Type I IFN inhibition is not universal for LACV as others have reported production of IFN-β in the presence of LACV NSs in cell types such as myeloid dendritic cells and microglial cells (Kallfass et al., 2012; Taylor et al.,2014).

Summary of Thesis Work

We propose a model addressing the early interactions of LACV and host defense mechanisms as seen in Figure 1. When introduced into an animal host via a mosquito bite, the proboscis of an infected mosquito pierces skin capillaries, resulting in blood pooling into surrounding tissues as well as delivery of the virus to the tissue (Choumet et al. 2012). It is likely this interaction between arbovirus and blood represents one of the first interfaces between an arbovirus and innate immune factors. We examined the interactions of LACV and serum and present findings that suggest that factors in serum do not neutralize LACV, but rather unexpectedly, can potentiate LACV infectivity. This enhancement is observed in insect-derived LACV but not mammalian derived LACV.

Following this virus entry into the host, LACV can infect skin cells present at the site of entry to begin replication. Since the site of virus entry is through the skin, there has been strong interest in how dermal cell types, such as keratinocytes and fibroblasts, can play roles in the

outcome of these infections. We have examined the outcome of LACV infection of human keratinocytes cells in culture. Here, we show that keratinocytes are highly permissive to LACV infection and support rapid virus growth and extensive cell death. However, during multi-cycle LACV infections of keratinocytes, IFN responses can limit spread through the population of cells. Unexpectedly, we show that IFN-β induced by LACV infection also contributes to the killing of bystander non-infected neighboring cells.

From these results, we propose that host defense mechanisms can either promote or restrict virus entry and dissemination, and that the balance of these outcomes dictates the success of virus infection. This work contributes to the knowledge of arbovirus-host interactions which is key to understanding LACV pathogenesis.

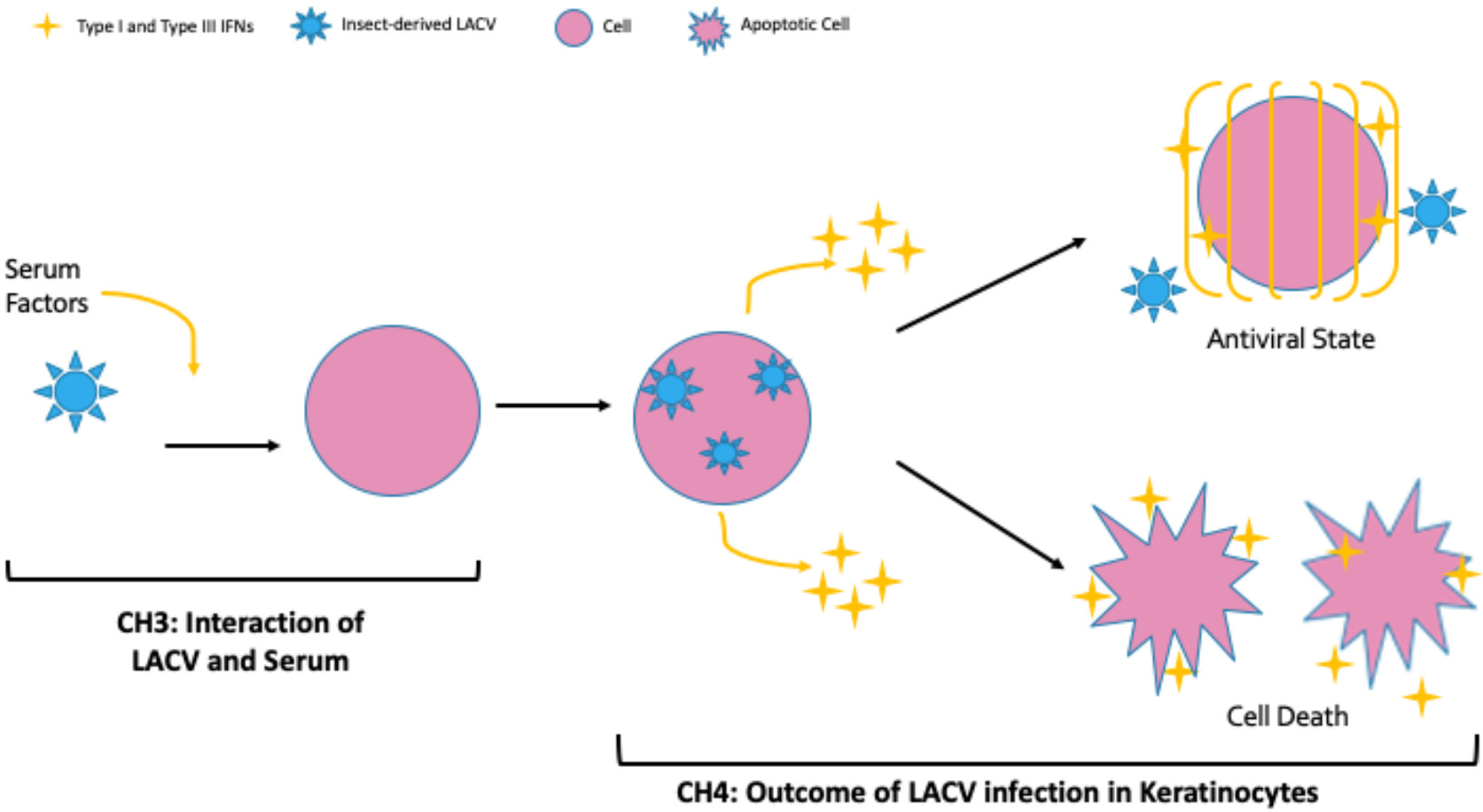


Figure 1: Model of early host defense mechanisms against LACV infection

Upon virus introduction by mosquitoes, LACV interacts with serum factors that enhance entry into cells. Dermal cells such as keratinocytes serve as sites of initial virus replication. LACV can productively infect keratinocytes. As a result of infection keratinocytes produce type I and type III interferons that act to restrict virus spread by inducing an antiviral state in nearby uninfected cells. Sustained antiviral signaling also results in bystander cell death of uninfected cells within a population

CHAPTER 2: MATERIALS AND METHODS

Cells, Viruses, and Infections

The HaCaT keratinocyte cell line was obtained from AddexBio Technologies Inc. (San Diego, CA, USA). The Hs27 fibroblast cell line (CRL-1634TM) was acquired from ATCC®. Vero cells were provided by Robert Lamb (Northwestern University, Evanston, IL, USA). Cultures of HaCaT, Hs27 and Vero cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated bovine calf serum (HI FBS, Hyclone, Logan, UT). La Crosse virus (LACV) was kindly provided by Andrew Pekosz (Johns Hopkins Bloomberg School of Public Health, Baltimore, MD).

Insect-derived LACV was grown in C6/36, with infections carried out in Leibovitz′s L-15 Medium (with 2% HI FBS) supplemented with 10% Tryptose broth (Thermo Fisher Scientific Waltham, MA, USA). After 1 hour (hr) incubation, cells were washed and replaced with Leibovitz′s L-15 Medium, without HI FBS, and 0.4% bovine serum albumin (BSA, Thermo Fisher Scientific,Waltham, MA, USA). LACV derived from human cells was grown in HaCaT cells maintained at passages below 10 to maintain consistency in culture. HaCaT Infections were performed at a multiplicity of infection (MOI) of 1 PFU/cell by incubating virus and cells in DMEM 2% HI FBS. After 1 hr of incubation, cells were washed, and media was replaced with DMEM 0% supplemented with 0.4% BSA. After 24hrs virus was collected. Human cell-derived LACV (HaCaT-LACV) underwent two rounds of passage in HaCaT before use in experiments.

Viral titers were determined by plaque assay on Vero cells. Briefly, 6-well plates of Vero cells at 90% confluency were infected with serial dilutions of LACV in DMEM supplemented with 10% BSA. After 1 hr incubation, cells were washed with PBS and overlayed with 1% agar in DMEM, 2% FBS, and 10 mM HEPES (pH 7.2) as described previously. For some experiments an overlay of a 1:1 mixture of 0.6% agarose and DMEM supplemented with 2% FBS was used to improve plaque clarity. After five days, overlay was removed and cells were fixed and stained with formaldehyde and a 0.1% crystal violet solution (Johnson et al., 2008.)

HaCaT cells were used at passages below 10 to maintain consistency in culture. Infections were performed at a multiplicity of infection (MOI) of 5, 0.5 or 0.05 plaque forming units (PFU)/cell by incubating virus and cells in DMEM containing 2% HI FBS. As a control, cells were also mock- infected by incubating with media only. After 1 hr of incubation, cells were washed, and media was replaced with DMEM 10% HI FBS. Hours post-infection (hpi) were counted from the time the virus was initially added to the cells.

Serum Incubation Assays

Assays were carried out as described previously (Johnson et al., 2008.). Briefly, a calculated number of PFU of I-LACV or HaCaT-LACV, was incubated at the indicated temperatures for times indicated in figure legends with dilutions of normal human serum (NHS), heat-inactivated serum (HI), serum depleted of human immunoglobulins (Celprogen, Torrence, CA, USA), or mammalian serum. Virus incubated with BSA or alone were used as controls as indicated on the figure legends. After incubation, viral titers were determined by plaque assays as described above.

Alternatively, serum- or control-treated LACV samples were used to infect Vero cells in 48-well well dishes. At 4 hrs post-infection, cells were fixed in formaldehyde, permeabilized and stained with LACV N rabbit polyclonal sera and Alexa Fluor® 568 (Invitrogen, Carlsbad, CA, USA) as described previously (Young et al., 2006). Cells were imaged at 10X with a Keyence All-in-One Fluorescence Microscope BZ-X800 (Keyence America, Itasca, IL, USA) and cell numbers determined using the IncuCyte® Live-Cell Analysis System (Essen Bioscience, Ann Arbor, MI, USA) and cell-by-cell software module.

Western Blotting

Treated LACV samples, or HaCaT cells plated at 4×10^4 cells/well in a 24-well plate and infected with LACV at a MOI of 5, were lysed in 1% sodium dodecyl sulfate (SDS) at the indicated timepoints. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting with rabbit polyclonal sera against the N protein of LACV at 1:5000 dilution. or with an antibody against human IgG (Millipore Sigma, Burlington, MA, USA) at 1:1000 dilution. Polyclonal anti-N serum was produced using N protein expressed in bacteria from PCR fragments generated from the LACV genome. The full protocol is available upon request. Blots were visualized by horseradish peroxidase-conjugated antibodies and chemiluminescence (Thermo Fisher Scientific).

IgG Depletion

Normal Human serum or HI serum was incubated with an equivalent amount of Recombinant Protein G - Sepharose™ 4B beads (Invitrogen) or with unconjugated Sepharose

beads as a control for 20 minutes on ice. Reactions were spun down and supernatant removed. Sequential incubation with Sepharose-Protein G or control beads was carried out three times for each serum sample to ensure a complete depletion. To confirm depletion, samples were analyzed by SDS-PAGE, followed by western blotting with an antibody specific for human IgG (Millipore Sigma)

Centrifugation Assays

To purify Insect-derived LACV, virus grown as indicated above was concentrated by centrifugation through a glycerol cushion (4 hrs; 24,000 rpm; SW28 rotor), and resuspended virus was further purified by centrifugation on a 20–60% sucrose gradient (1 hr; 38,000 rpm; SW41 rotor). The virus band was collected, pelleted as a above, and resuspended in DMEM. Purified I-LACV was incubated alone or with normal human serum (NHS) at a ratio of 1:1 (vol/vol) for 1hr at 37°C. Samples were layered on top of 20 to 60% sucrose gradients and subjected to ultracentrifugation at 42,000 rpm, for 1 hr, in an SW55 rotor at 4°C. Fractions were collected from the bottom of the tube (250 µl) and were analyzed SDS-PAGE followed by western blotting with LACV N rabbit polyclonal sera or with a monoclonal rat antibody specific for human IgG (Millipore Sigma)

Cell Viability and Caspase Assays

Cells cultured in 24-well plates or 48-well plates were treated as indicated in each figure legend. Media and trypsinized cells were centrifuged and analyzed for propidium iodide (BD Bioscience) staining as described by the manufacturer. Cells were analyzed by flow cytometry

using the CytoFLEX (Beckman Coulter, Brea, CA, USA) and 10,000 independent events were analyzed using CytExpert software (Beckman Coulter).

Alternatively, cytotoxicity assays were performed in 96-well white plates (Corning, Corning, NY, USA) using CytoTox-Glo reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are expressed as a fold change over mock-infected cells analyzed in parallel. Functional caspase assays were performed in 96-well white plates (Corning) using Caspase-Glo 9, 8, or 3/7 assays (Promega) according to the manufacturer's instructions. Data are expressed as a fold change over mock-infected cells analyzed in parallel.

Z-VAD-FMK Treatment

Cells were plated at 3.5×10^4 cells/well in a 24-well plate and treated with Z-VAD-FMK (Promega) or DMSO as a vehicle control diluted in DMEM supplemented with 10% HI FBS at a concentration of 40 μM for 30 min. Cells were then infected as described above. Virus was removed and replaced with Z-VAD-FMK at 20 μM diluted in DMEM supplemented with 10% HI FBS.

Supernatant Preparation

At 80% confluency, 6-well plates of cells were infected with LACV at a MOI of 5 PFU/cell. At times indicated in the figure legends, media was collected and treated with UV light from a germicidal G30T8 bulb for 15 min to inactivate LACV. To confirm virus inactivation, naïve HaCaT cells were treated with UV-supernatant. After 24 h, cells were trypsinized, washed, fixed, and permeabilized (eBioscience, San Diego, CA, USA) according to

manufacturer's protocol. Cells were stained with an anti-LACV Gc antibody 807.31ab (kindly provided by Andrew Pekosz) and Alexa Fluor® 488, a fluorophore conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Cells were analyzed by flow cytometry using the CytoFLEX system (Beckman Coulter), and 10,000 independent events were recorded and analyzed by using CytExpert software.

Reverse Transcription and Real-Time PCR

HaCaT cells cultured in 6-well dishes were infected at a MOI of 5 PFU/cell. At timepoints indicated in the figure legends, cells were collected in TRIzol® followed by RNA extraction (Invitrogen). To produce cDNA, 1 µg of total RNA was used with TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) as described in the manufacturer's instructions. Quantitative real-time PCR was performed using Bio-Rad CFX Connect Real-Time (Bio-Rad, Hercules, CA, USA) and Fast SYBR® FAST Green Master Mix (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined using CFX Manager 3.1 Software (Bio-Rad) and the following primers (Table 1):

Table 1: List of Primers

	Forward Primer	**Reverse Primer**
β-actin	5′-GATCATTGCTCCTCCTGAGC-3′,	5'-ACTCCTGCTTGCTGATCCAC-3,
IFN-β	5'-CAGCTCTTTCCATGAGCTACAA-3'	5'-CAGTATTCAAGCCTCCCATTCA-3'
IFN-λ1	5'-CTTGGACCGTGGTGCTG-3'	5'-CAGCCCTTCCCAGTTGTG-3'
IFN-λ2/3	5'-CAGTGCTGGTGCTGATG-3'	5'-GACTTGAACTGGGCTATGTG-3'

Interferon, Ruxolitinib and Neutralizing Antibody Treatment

HaCaT cells plated at 3×10^4 cells/well were treated with varying concentrations of IFN-Lambda (IL-29 or IFN-λ1) or IFN Beta (IFN-β) (PBL Assay Sciences, Piscataway, NJ, USA) for 16 h. All dilutions were carried out in DMEM supplemented with 10% HI FBS.

HaCaT cells were plated at 4×10^4 cells/well in a 24-well plate and treated with DMSO as vehicle control or Ruxolitinib (Invivogen, San Diego, CA, USA) at a concentration of 1 μM for 16 h. Cells were infected for 1 h before washing and replacement with DMEM supplemented with 10% HI FBS and 1 μM Ruxolitinib or DMSO vehicle control. Alternatively, cells were infected and then treated with neutralizing antibodies against IL29 (Invivogen), IL28a or IFN-λ2 (Invivogen), and Human IFN-λ1 receptor (IFNLR; PBL Assay Science), or IFN-β (Millipore, Burlington, MA, USA) at the concentrations stated in the figure legends. Corresponding isotype antibodies were used as negative controls.

Cell Viability and Virus Infection Quantification

HaCaT cells were cultured in a 24-well plate. Media and trypsinized adherent cells were centrifuged and stained with Zombie RedTM (BioLegend, San Diego, CA, USA) to quantify viability. Cells were then fixed, permeabilized (eBioscience) and stained with anti-LACV Gc antibody 807.31ab (kindly provided by Andrew Pekosz) and Alexa Fluor® 488 (Invitrogen). Cells were analyzed by flow cytometry using the CytoFLEX system (Beckman Coulter), and 10,000 independent events were recorded and analyzed by using CytExpert software.

Statistical Analyses

Values are the mean of three replicates and experiments were performed at least twice. Statistical analysis was performed using GraphPad Prism 8, student's *t*-test or a two-way ANOVA. In all figures, * indicates p-value < 0.05, ** indicates p-value < 0.01, and *** indicates p-value < 0.001.

CHAPTER 3: ENHANCEMENT OF INFECTIVITY OF INSECT CELL-DERIVED LA CROSSE VIRUS BY HUMAN SERUM

Serum Stabilizes and Enhances Infectivity of Insect Cell- Derived LACV

Human sera was analyzed for the ability to neutralize LACV derived from C6/36 insect cells, termed I-LACV. As shown in Figure 2A, LACV was grown in C6/36 insect cells in the absence of any serum and the resulting titer of the stock was calculated by plaque assay on Vero indicator cells. In this assay, 100 calculated PFU of I-LACV was incubated for 1 hr at 37°C alone, with a 1:10 dilution of NHS as a source of complement and antibodies, or heat-inactivated (HI) serum as a control with no viable complement activity. Sera from four different donors was used to ensure that results were not due to donor variability. Remaining infectivity was determined by plaque assays on Vero indicator cells and was expressed as a percentage of the expected input PFU. As seen in Figure 2B, there was no observed neutralization of LACV infectivity by either by NHS or HI serum (grey and hatched bars), indicating that neither complement in NHS nor antibodies in serum neutralized I-LACV. Unexpectedly, in samples incubated alone (black bars, Figure 2B), there was only approximately 10% recovery of the expected 100 PFU of I-LACV, while I-LACV samples that were treated with either NHS or HI serum showed an increase in the number of recovered PFU (light grey and hatched bars, Figure 2B). When expressed as a percentage of expected PFU, Figure 2B shows that incubation with serum from donor K yielded 150% more than the expected PFU and donor L had 200% more PFU as compared to the expected PFU. There was no significant difference in the enhanced recovery of PFU between NHS and

serum that had been heat-inactivated. In the experiments shown below, the enhancement of infectivity varied between donors and between experiments in the range of 200% to 400%.

To determine if the observed enhancement was dose-dependent, 200 PFU of I-LACV was incubated for 1 hr at 37°C either alone or with ten-fold dilutions of NHS before determining remaining infectivity by plaque assay. As shown in Figure 2C, I-LACV alone resulted in ~10% of expected infectivity. Enhancement of infectivity of I-LACV by serum was dose-dependent, with 1:10 dilution of HI serum enhancing infectivity by ~200% as compared to expected PFU and 1:100 dilution having a ~130% enhancement over expected PFU. At dilutions greater than 1:1000, there was minimal effect.

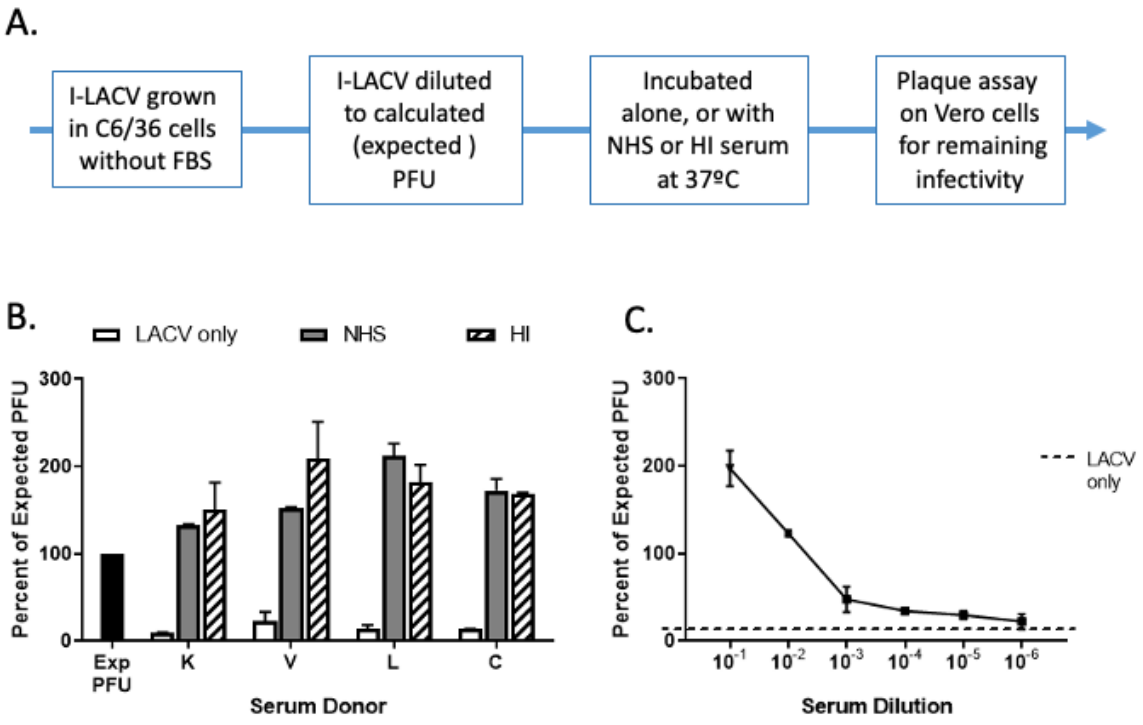


Figure 2: Normal human serum and heat-inactivated serum enhance I-LACV infectivity.

(A) Experimental timeline to test enhancement of infectivity of I-LACV by serum. (B) 200 PFU of I-LACV was incubated with DMEM as a control (LACV only, white bars) or with 1:10 dilution of NHS (grey bars) or 1:10 dilution of HI serum (striped bars) from four different donors indicated by letters at 37°C for 1 hr. Remaining infectivity was determined by plaque assays. Expected PFU (Exp PFU, black bar) represents calculated input virus. (C) 200 PFU of I-LACV was incubated alone (dashed line) or with the indicated dilutions of HI serum from a single donor at 37°C for 1 hr. Remaining infectivity was determined by plaque assays. Values are the mean of three replicates with error bars indicating standard deviation.

In the above experiments, virus incubated alone had significantly less infectivity compared to the expected PFU. We hypothesized that I-LACV loses infectivity during incubation without the presence of carrier protein. To test this, a time-course of incubation with human serum was carried out where 200 PFU of I-LACV was incubated for various times at 37°C with a 1:10 dilution of HI NHS or with 1:10 dilution of BSA as a carrier protein control. Remaining infectivity was determined by plaque assays. As seen in Figure 3A, I-LACV in the presence of BSA showed a time-dependent loss of infectivity starting as early as 15 min and by 60 min only ~10% of expected infectivity was detected. By contrast, HI NHS maintained the same infectivity from 2-45 minutes at about ~350% over the expected PFU. In this particular experiment, infectivity of I-LACV incubated with serum returned to 100% at later 60 minutes, but this was dependent on the serum donor.

To determine the effect of temperature on changes in infectivity, 200 PFU of I-LACV was incubated alone, with BSA as a control or with HI serum at 4°C, 25°C or 37°C for 1 hr before

plaque assay on Vero cells. I-LACV incubated with HI serum showed an increase of ~300% infectivity over the expected PFU at all temperatures tested (Figure 3B). I-LACV incubated alone or with BSA at 4°C had ~70% infectivity as compared to expected PFU while at 25°C and 37°C infectivity had dropped to ~9% of the expected PFU.

To determine if I-LACV enhancement and stabilization was specific to human serum, 200 PFU of I-LACV was incubated at 37°C for 1 hr either alone, with BSA as a control or with HI serum from different species. All tested sera showed some level of enhancement over expected PFU with I-LACV incubated with human (Hu) serum having the highest levels of enhancement at ~300% infectivity over the expected PFU. Primate (P) and chicken (C) had the lowest increase in infectivity, ~150% above the expected PFU (Figure 3C). As seen in previous experiments, I-LACV incubated either alone (DMEM) or with BSA had ~15% infectivity as compared to expected PFU.

Taken together these data suggests that I-LACV is unstable when incubated alone and a control carrier protein has little effect on stability. In contrast, human serum has heat-stable components that have two different effects on I-LACV: 1) Stabilization of LACV infectivity seen by the reduction of infectivity in virus only samples as compared to expected PFU, and 2) Enhancement of infectivity as seen by the increase in number of PFU of LACV incubated with HI as compared to expected PFU. Furthermore, heat-stable components responsible for enhancement are common to a broad collection of animal sera. In the following sections we attempt to define these heat-stable components.

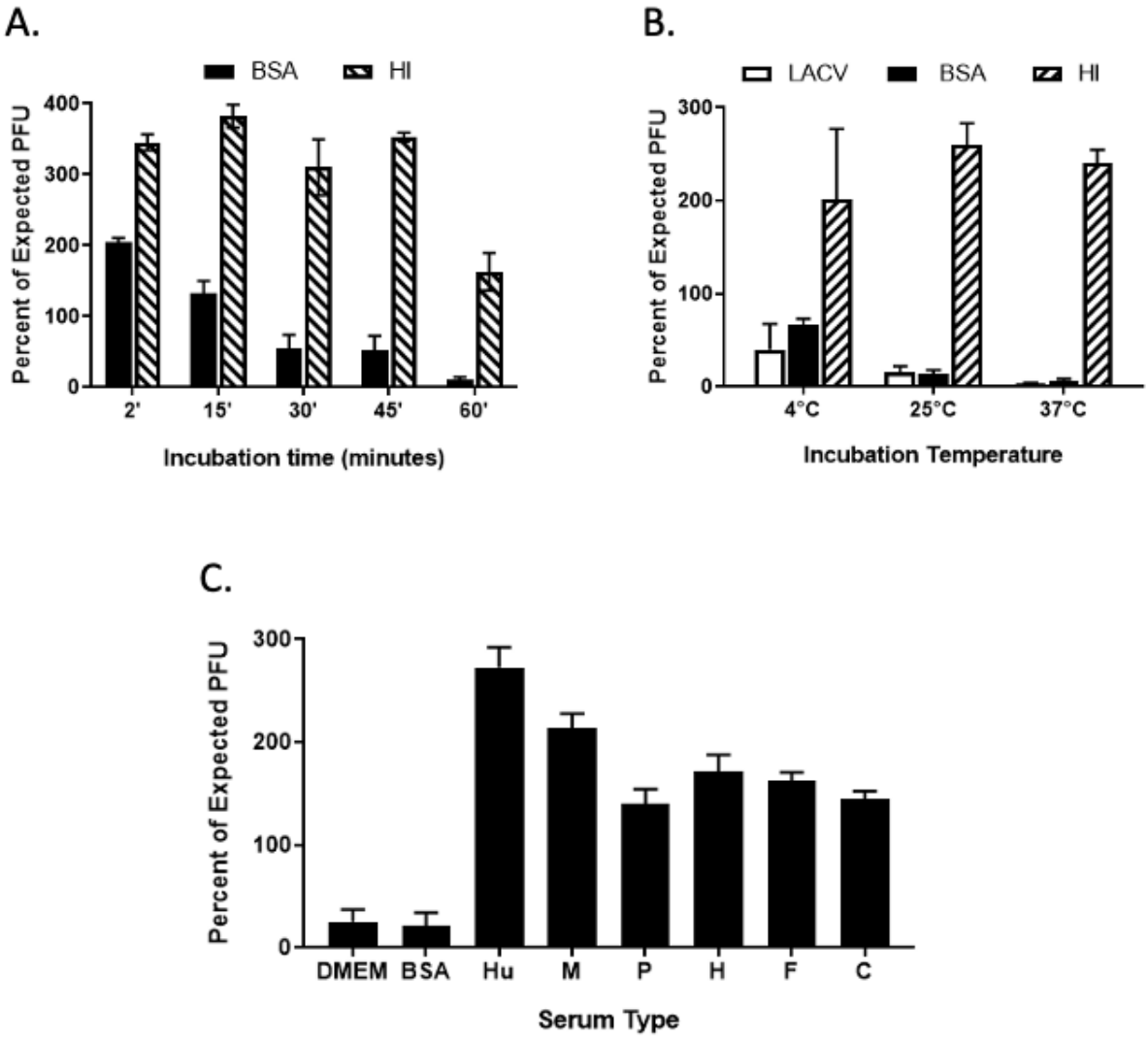


Figure 3: A carrier protein cannot stabilize or enhance I-LACV infectivity

(A and B) 200 PFU of I-LACV was incubated with a 1:10 dilution of BSA (black bars) as a control or with 1:10 dilution of HI serum (striped bars) at 37°C for the indicated times (A) or for 1 hr at the indicated temperatures (B). Remaining infectivity was determined by plaque assays. (C) 200 PFU of I-LACV was incubated alone, with a 1:10 dilution of BSA as a control, or with a 1:10 dilution of HI serum from different species at 37°C for 1hr. Remaining infectivity was determined by plaque assays. Abbreviations denote Hu as human, M as mouse, P as primate, H as horse, F as

ferret, and C as chicken. Values are the mean of three replicates with error bars indicating standard deviation.

Serum Enhances I-LACV Infectivity at Early Steps of the Virus Life Cycle

The readout in the above enhanced infectivity experiments was an increase in number of detected plaques in a sample. Since plaques are the result of multiple rounds of replication, they are not able to determine the stage of the virus life cycle which is affected by incubation with serum. As an alternative approach, I-LACV was incubated alone, with BSA, or with HI human serum for 15 minutes at 37°C and samples were used to infect monolayers of Vero cells. After 4 hrs of infection, cells were fixed, permeabilized and number of LACV infected cells was detected by immunofluorescence staining with a polyclonal sera against LACV N. This time post infection was chosen to ensure that replication was limited to only those cells that were infected with the input virus (e.g. Cruz and Parks, 2020). As shown in Figure 4A and quantified in Fig 4B, Vero cells infected with I-LACV that had been treated with HI serum showed about ~3500 infected cell foci per tissue culture well. By comparison, cells infected with untreated I-LACV, or with virus treated with BSA had much lower number of infected cell foci per tissue culture well at ~100 and ~150 cells, respectively. Together, these data support the prior findings that NHS treatment leads to enhancement of I-LACV infectivity and that enhancement occurs at an early step in virus replication.

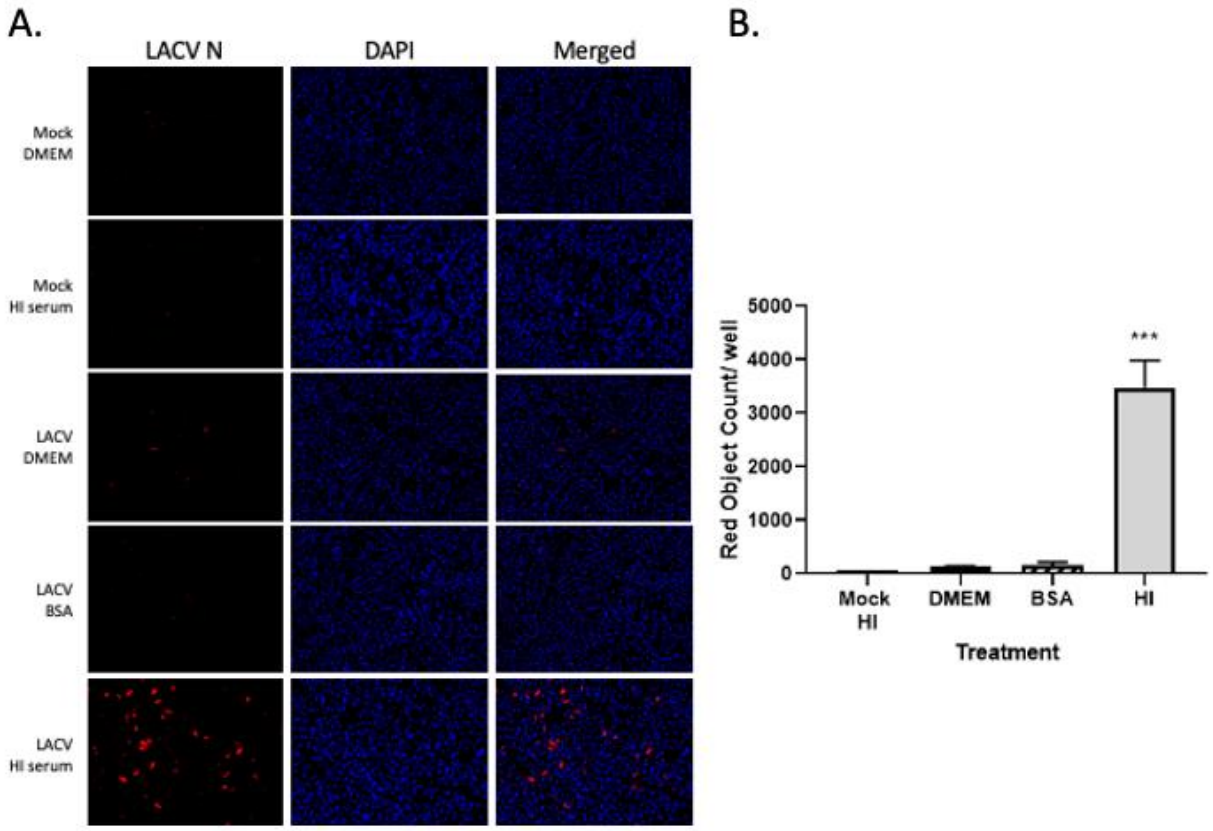


Figure 4: HI Serum enhances LACV infection at early steps in the virus replication cycle

(A and B) Vero cells were infected with I-LACV that had been incubated with DMEM (white bars) or with a 1:10 dilution of BSA (black bars) as control, or with HI serum (striped bars) at 37°C for 15 mins. Immunostaining for the LACV N protein and DAPI staining was performed 4 hrs post infection and imaged at 10X magnification. Mock infected cells treated with HI were included as a control (B). Number of cells stained with antibody to LACV N was determined using the Incucyte® Analysis Software. Values are the mean of three replicates with error bars indicating standard deviation and *** indicating *p*-values of < 0.001.

Stabilization and Enhancement of I-LACV Infectivity is Removed When IgG is Depleted from Serum.

To determine if antibodies present in the serum contributed to enhanced infectivity, HI human serum was treated with protein G-sepharose beads to deplete IgG antibodies. Western blotting confirmed that IgG had been depleted by protein G-sepharose treatment but not by control unconjugated sepharose treatment (Figure 5A). Treated serum was incubated with 300 PFU of I-LACV at 37°C for 1 hr, with unprocessed HI serum and HI serum treated with unconjugated sepharose beads used as controls. Remaining infectivity was determined by plaque assays. Protein G-treated serum resulted in a statistically significant decrease in infectivity, with ~150% over expected PFU (black bar) as compared to serum treated with unconjugated sepharose (striped bar) or untreated HI serum (grey bar) at about 300-400% over expected PFU (Figure 5B). This result suggests that IgGs are involved in the enhancement of I-LACV infectivity. It is noteworthy that despite the significant decrease in infectivity, the protein G sepharose treatment was not enough to bring infectivity down to same levels as the virus-only control (white bar). This suggests that there might be other serum factors involved in I-LACV stabilization and infectivity.

To test the hypothesis that IgG interacted directly with I-LACV, 100 ug of sucrose-purified I-LACV was incubated alone or with HI human serum at 37°C for 1 hr and samples were sedimented through a 20-60% sucrose gradient. A sample of serum alone was also analyzed by gradient sedimentation as a control. After sedimentation, gradient fractions were collected and analyzed by western blotting with polyclonal sera against LACV N or with antibody specific for IgG. As shown in Figure 5C, I-LACV treated alone for 1 hr was found to sediment as a broad peak that was spread between fractions 4-10, consistent with heterogeneous degradation of virions. By

contrast, I-LACV incubated for 1 hr with HI serum was found as a sharp peak that sedimented into a single fraction, consistent with a homogeneous virus population. Furthermore, analysis of fractions with anti-IgG showed a distinct band that sedimented to the same fraction as I-LACV virions, which was not seen in samples of HI serum alone. Together, these data support the contention that human serum stabilizes I-LACV and IgG can be found to associate with I-LACV to a limited extent.

To determine if other antibody types in serum could also contribute to the enhanced infectivity, a time-course of incubation was carried out with commercially available human serum depleted of all immunoglobulins (Ig-depleted). Here, 300 PFU of I-LACV were incubated alone, with BSA, with HI serum, or with Ig-depleted serum at 37°C for the timepoints indicated in Figure 5D. Plaque assays were used to determine remaining infectivity. I-LACV treated with Ig-depleted serum had a similar infectivity profile to virus incubated with BSA, with virus infectivity decreased from about 50-70% of expected PFU after 2 mins of incubation down to undetectable levels by 60 mins. I-LACV incubated with HI serum remained constant over the time-course at ~400% over expected PFU.

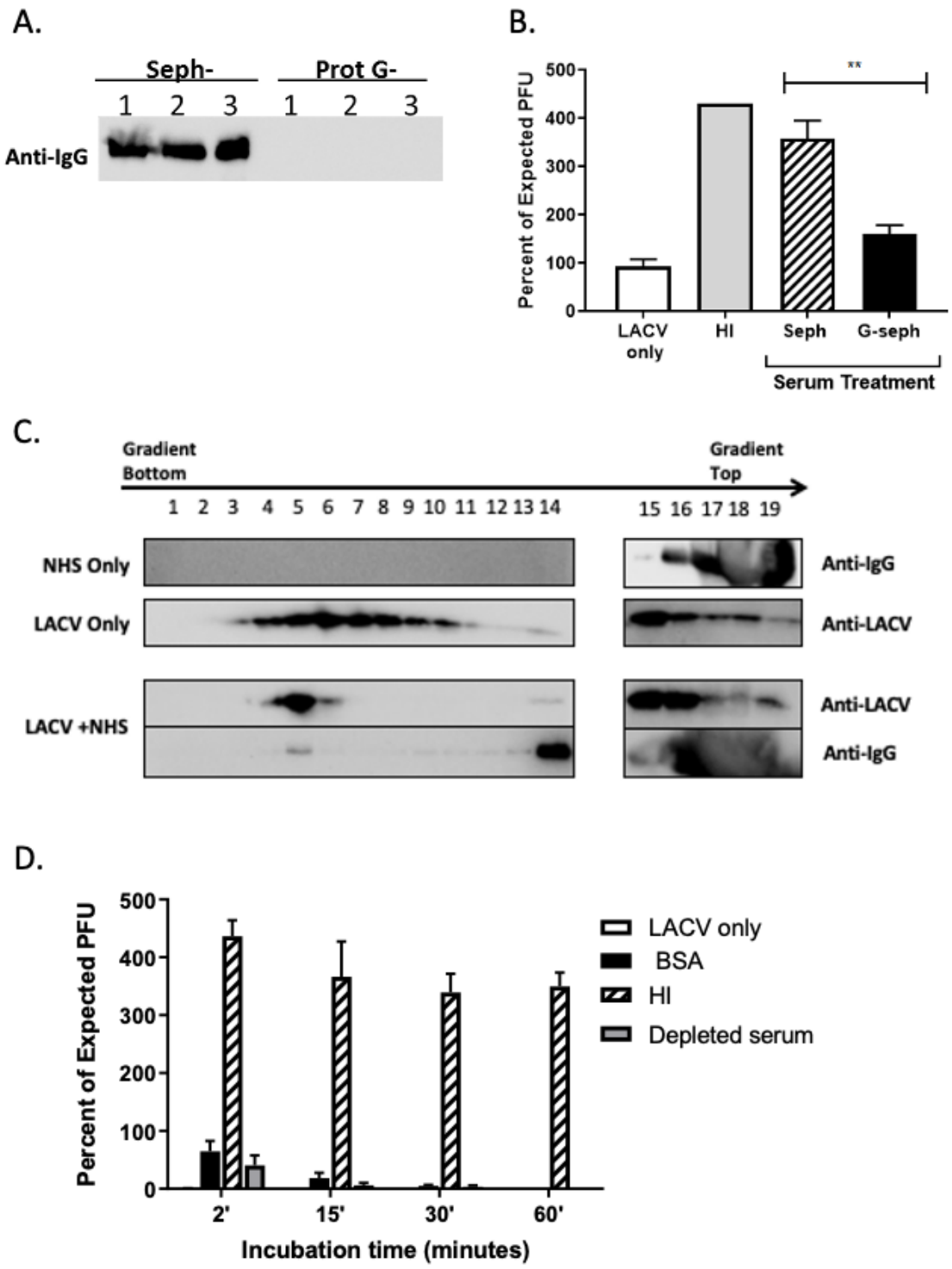


Figure 5: Depletion of IgG from HI serum reduces the ability of HI serum to enhance I-LACV infection.

(A) HI serum was incubated with sepharose alone as a control or with protein G-sepharose at 25°C for 20 minutes for IgG depletion. After three sequential rounds of depletion, treated serum samples were lysed and analyzed by Western blotting for human IgG. (B) 300 PFU of I-LACV was incubated for 1 hr at 37°C with DMEM (white bars) or with a 1:10 dilution of either HI (grey bars), HI treated with sepharose as a control (striped bars) or HI treated with Protein G-sepharose (black bars). Remaining infectivity was determined by plaque assays. (C) Purified I-LACV was incubated alone or with NHS for 60 min at 37 °C and then analyzed by centrifugation through 15–60% sucrose gradients. Serum alone was included as a control. Fractions were collected from the bottom of the tube and analyzed for viral proteins by western blotting with antiserum specific for the LACV N protein (middle and bottom panels). Fractions from the NHS only sample and from NHS-treated virions were also analyzed by western blotting for the presence of IgG (top and bottom panel). (D) 300 PFU of I-LACV was incubated with DMEM (white bars), or with a 1:10 dilution of BSA (black bars) as control, with 1:10 dilution of HI serum (striped bars) or 1:10 commercial Antibody-depleted serum (grey bars) at 37°C for the indicated times. Remaining infectivity was determined by plaque assays. Values are the mean of three replicates with error bars indicating standard deviation and ** indicating *p*- values of < 0.01.

Infectivity of LACV Derived from Human Keratinocytes is not Altered by Incubation with Human Serum

We tested whether the infectivity of LACV derived from human cells was also affected by incubation with NHS. LACV was grown in the absence of serum through two cycles of low MOI

infection in the human keratinocyte cell line HaCaT (HaCaT-LACV). A calculated 100 PFU of either HaCaT-LACV or I-LACV was incubated alone, with BSA, or with a 1:10 dilution of NHS serum or HI serum for 1 hr at 37°C and remaining infectivity was determined by plaque assay. As shown previously, I-LACV incubated alone or with BSA showed a loss of infectivity to ~10% of expected levels, and this was enhanced to ~250-300% by incubation with NHS or HI (Figure 6A). By sharp contrast, HaCaT-LACV showed no increase or decrease in expected infectivity of 100 PFU by any of these incubations. These data also indicate that complement and/or antibodies present in NHS do not have an effect on HaCaT-LACV infectivity.

To extend these results, we tested the ability of serum from a range of donors to enhance HaCaT-derived LACV infectivity (Figure 6B). Two of the three tested donor sera had no significant effect on infectivity, consistent with data in Figure 6A. For one donor, there was a slight enhancement in HaCaT-LACV infectivity, but this enhancement was substantially lower that that seen with I-LACV. Together these data indicate that LACV derived from human keratinocytes differs from I-LACV in its stability and enhancement by serum-derived components.

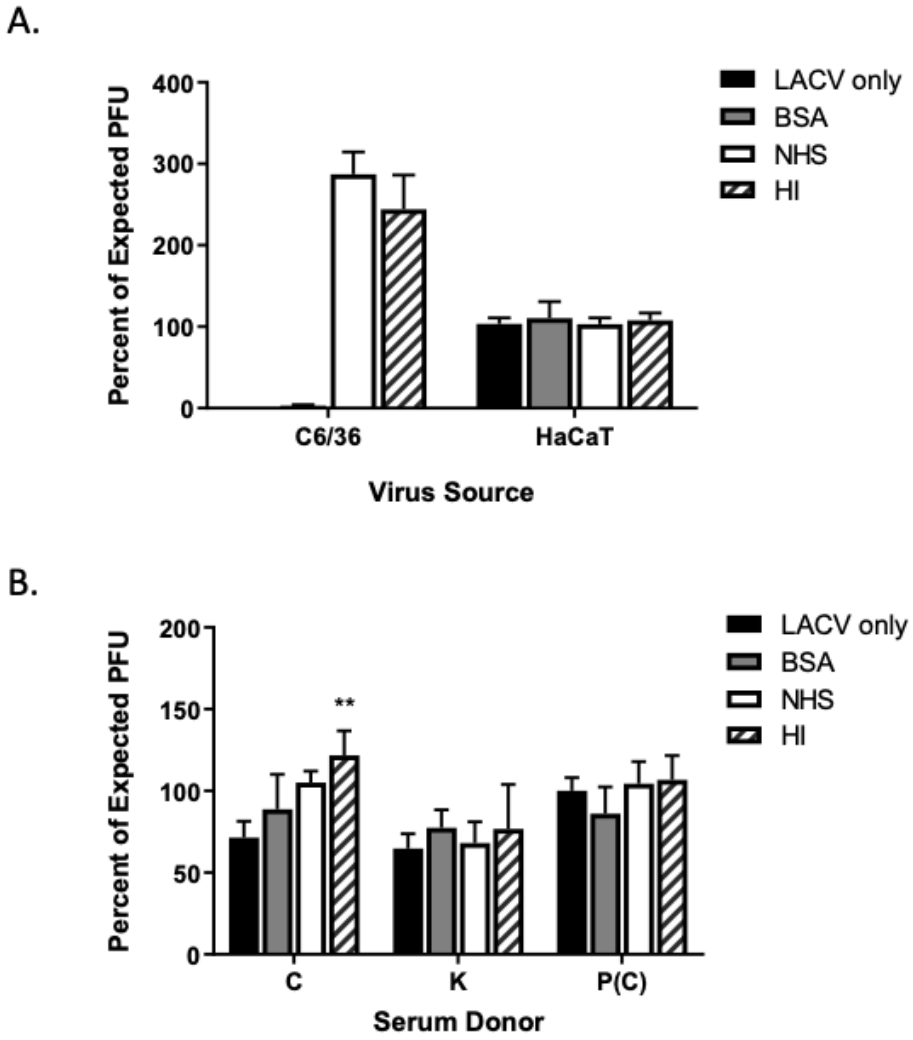


Figure 6: Infectivity of HaCaT-LACV is stable and is not enhanced by human serum

(A) 100 PFU of I-LACV derived from C6/36 cells or LACV from HaCaT cells was incubated with DMEM, a 1:10 dilution of BSA as control, or with a 1:10 of NHS or HI serum at 37°C for 1 hr before determining remaining infectivity by plaque assay. (B) 100 PFU of LACV derived from HaCaT cells was incubated with DMEM or a 1:10 dilution of BSA as controls, or with a 1:10 dilution of NHS or HI serum from three different donor at 37°C for 1 hr. Remaining infectivity

was determined by plaque assays Values are the mean of three replicates with error bars indicating standard deviation and ** indicating *p*-values of <0.01.

CHAPTER 4: LA CROSSE VIRUS INFECTION OF HUMAN KERATINOCYTES LEADS TO INTERFERON-DEPENDENT APOPTOSIS OF BYSTANDER NON-INFECTED CELLS IN VITRO

HaCaT Cells are Permissive to LACV Infection

To determine if LACV can productively infect human keratinocyte cells, the HaCaT cell line was infected with LACV at a MOI of 5 PFU/cell. At 12, 24, 48 and 72 h post-infection (hpi), cell supernatant was collected and viral titers were quantified by plaque assay. As shown in Figure 7A, viral titers increased rapidly over time with a peak titer of ~10^6 PFU/mL at 24 hpi. When earlier timepoints were examined, virus production was detected by 6 hpi and had peaked at 10^6 PFU/mL by 14 hpi. HaCaT cells were infected at a high MOI and viral protein expression was measured by Western blot analysis of LACV nucleocapsid protein (N) accumulation. As shown in Figure 7B, N protein was detected in lysates as early as 6 hpi. As an alternative assay, HaCaT cells were mock- infected or LACV-infected at a MOI of 5 PFU/cell, and cells were collected at 8, 12, and 16 hpi for analysis of total viral Gc expression by flow cytometry using an antibody against the LACV Gc protein. Consistent with LACV N expression, almost 90% of LACV-infected cells expressed the Gc glycoprotein by 8 hpi with no substantial increase at later timepoints (Figure 7C). Together, these data indicate that HaCaT cells are permissive to high multiplicity LACV infection and can produce high levels of progeny virions.

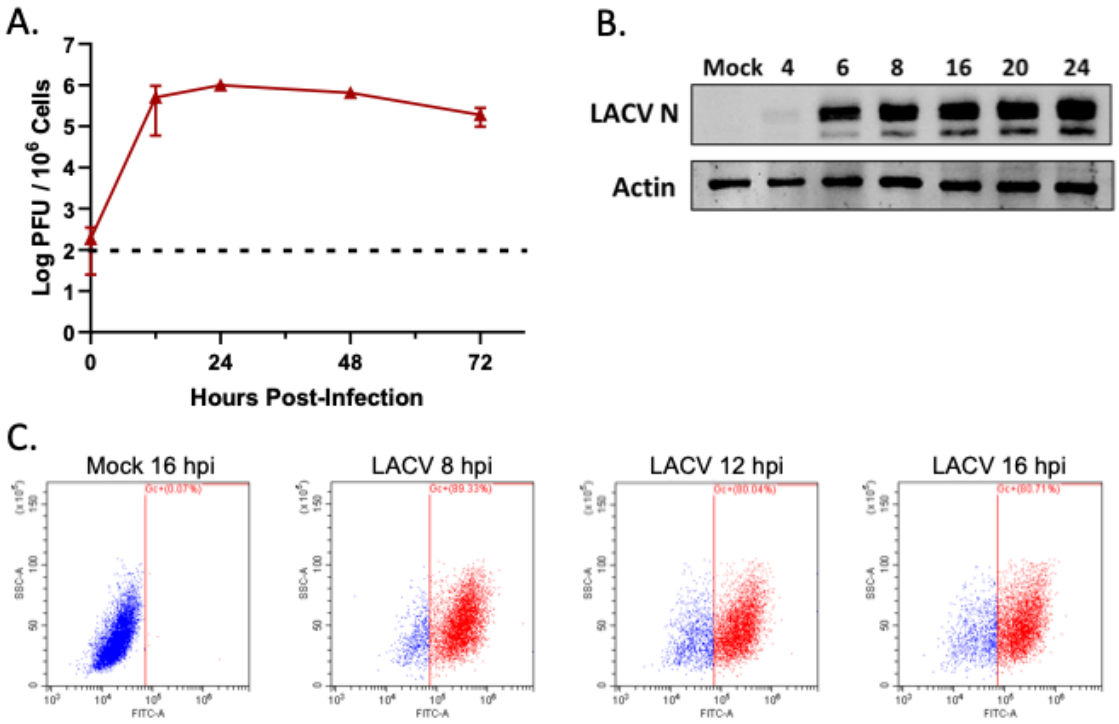


Figure 7: LACV can productively infect human keratinocyte cells.

HaCaT cells were infected or mock- infected with LACV at a MOI of 5 PFU/cell. (A) At the indicated hpi, media was collected and viral titers were determined by plaque assay. Dotted horizontal line indicates limit of detection. (B) At the indicated timepoints, cells were lysed and analyzed by Western blotting for β-actin or for LACV N or (C) cells were analyzed by flow cytometry for LACV Gc expression. Dot plots shown are representative of three replicates.

LACV Infection Induces Caspase-Dependent Cell Death in HaCaT cells

To examine LACV-induced cytopathic effects, HaCaT cells were infected at a MOI of 5 PFU/cell and visualized by microscopy. As shown in Figure 8A, cell rounding and detachment from the culture dish were evident as early as 8 hpi, concurrent with the kinetics of LACV N

protein expression (Figure 7B). To quantitate cell death, HaCaT cells infected at a high MOI were stained with propidium iodide (PI) at different times post-infection and analyzed by flow cytometry. As shown in Figure 8B, there was no significant PI staining at 8 hpi, however, by 16 hpi, ~30% of cells were PI-positive and this increased to ~40% by 20 hpi. A Cytotox-Glo assay (which measures release of a dead-cell protease) showed similar cytotoxicity at 24 hpi as seen with PI staining and by 36 hpi that toxicity had increased to 70% (Figure 8C). These results indicate that high MOI LACV infection induces significant cell death in HaCaT cells which correlate with the appearance of viral protein in the cells.

To determine if caspases are activated in HaCaT cells following LACV infection, cells were infected at a high MOI and caspase activity was measured in vitro by Caspase-Glo assays. As shown in Figure 8D, the activity of effector caspases 3/7 were upregulated at 24 hpi to ~10-fold over mock- infected control cells. Likewise, activities of both initiator caspase-8 and caspase-9, were upregulated to ~8-fold at 24 hpi by LACV infection as compared to mock-infected cells. To directly test the role of caspases in LACV-induced cell death, HaCaT cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK prior to infection at high MOI. At 24 hpi, cell viability was quantitated by PI staining and flow cytometry analysis. Infected cells treated with Z-VAD-FMK had significantly less PI staining, only ~20% of the cells stained positive as compared to 70% in the DMSO control (Figure 8E). Together, these data support the conclusion that LACV induces significant levels of death in HaCaT cells, and death is predominantly mediated by caspase-dependent pathways.

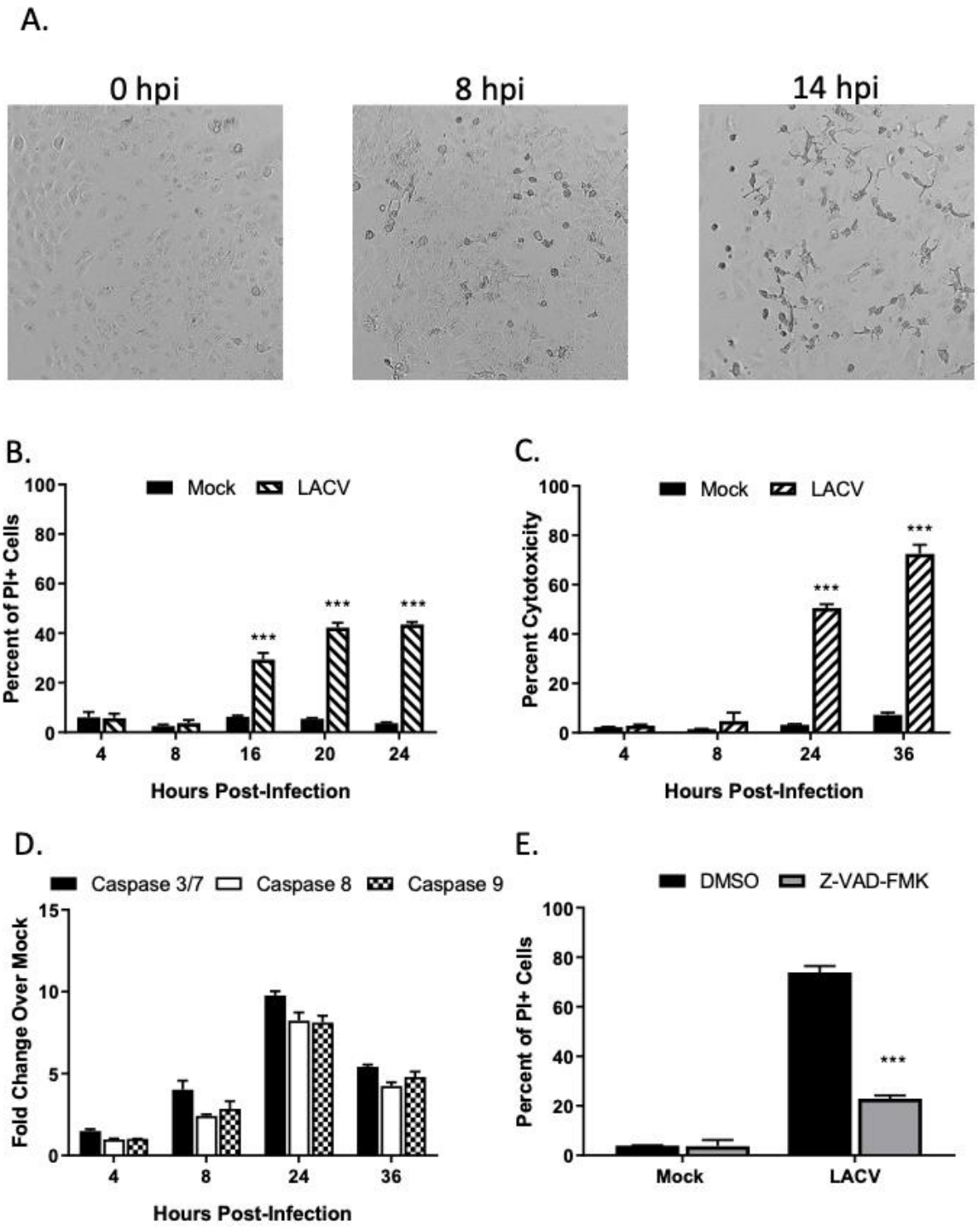


Figure 8: LACV infection of human keratinocytes induces caspase-dependent cell death.

(A–D) HaCaT cells were infected at a MOI 5 PFU/cell. (A) At the indicated hpi, cells were imaged at 10X magnification, and representative bright field images are shown. Alternatively, cell viability and cytotoxicity was determined by (B) PI staining or (C) Cytotox-Glo assay. (D) Caspase activity in cell lysates was determined by Caspase-Glo-3/7, -9 or -8 assays. (E) HaCaT cells were pre-treated with DMSO or with 40 μM of the pan-caspase inhibitor Z-VAD-FMK for 30 min. Cells were then infected at a MOI of 5 PFU/cell. After 24 h incubation with DMSO or with Z-VAD-FMK, cell viability was determined by PI staining. Values are the mean of three replicates with error bars indicating standard deviation and *** indicating *p*-values of < 0.001.

Multi-Cycle Spread of LACV Infection Is Restricted in HaCaT Cells

The above data show that HaCaT cells were permissive to LACV infection at a high MOI. To determine if HaCaT cells supported multi-cycle spread of LACV, HaCaT cells were infected at three different MOIs—a high MOI of 5 PFU/cell as baseline for infection, an intermediate MOI of 0.5 PFU/cell, and a low MOI of 0.05 PFU/cell. At indicated timepoints, cells were analyzed by flow cytometry for expression of the LACV Gc glycoprotein. As shown in Figure 9A, low MOI infection with LACV (0.05, striped bars) resulted in very little increase in percentage and number of Gc-positive cells over time, indicating that multi-cycle spread of LACV was restricted. Interestingly, HaCaT cells infected at the intermediate MOI of 0.5 (black bars) showed a distinct profile in the percentage and number of Gc-positive cells, with an initial 40% of the population being Gc-positive at 24 hpi, but a time-dependent decline in this value to ~10% by 72 hpi.

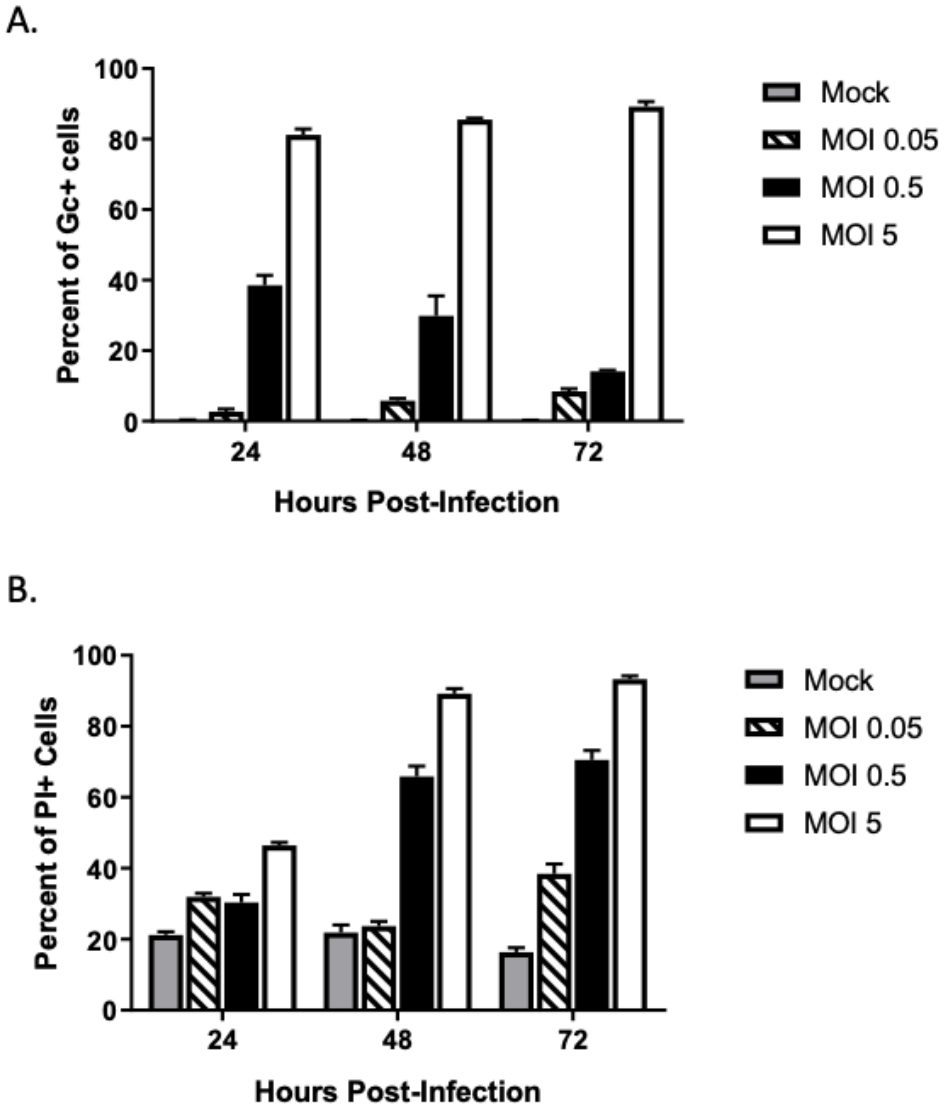


Figure 9: Restricted spread of LACV infection through a population of Keratinocytes

HaCaT cells were mock-infected or infected at a MOI of 0.05, 0.5 or 5 PFU/cell. (A) At the indicated hpi, cells were analyzed by flow cytometry for LACV Gc expression. Results are expressed as percentage of Gc-positive cells. (B) In parallel, cell viability was determined by PI staining. Values are the mean of three replicates with error bars indicating standard deviation.

In parallel, LACV-infected HaCaT death overtime at different MOIs was assayed using PI staining and flow cytometry. As shown in Figure 9B, mock-infected HaCaT cells had a consistent low-level PI staining (grey bars), whereas high MOI resulted in time-dependent increases in PI staining (white bars). The most striking result was seen with HaCaT cells infected at an intermediate MOI of 0.5 (black bars). While the percentage of Gc-positive cells decreased from 40% at 24 hpi to 10% at 72 hpi, there was a time-dependent increase in PI staining from 30% at 24 hpi to 70% hpi. These data indicate that at a MOI of 0.5 PFU/cell, there is a substantially greater increase in cell death as compared to the number of infected cells.

Together, the above data indicate two consequences of LACV infection of HaCaT cells: 1) LACV is restricted for multi-cycle spread within the HaCaT population, suggesting an antiviral response, and 2) there is increased cell death in the population as compared to the number of cells expressing Gc, suggestive of "bystander" cell death of non-infected cells. In the following sections, we individually address these two components of the HaCaT cell response to LACV infection.

Restriction in Spread Through a Population of HaCaT Cells is Due to an Antiviral Response Primarily Driven by Type I IFN

A media transfer experiment was used to determine if infected cells make products that restrict LACV infection of naïve cells. HaCaT cells were mock-infected or LACV-infected at a MOI of 5 PFU/cell. At 8 hpi, extracellular media was collected and exposed to UV light to inactivate virus. Successful inactivation of virus was evident by the lack of detection of LACV Gc glycoprotein when naïve cells were treated with UV-treated media (Figure 10A). In a

separate experiment, naïve HaCaT cells were treated with the UV-inactivated media for 16 h before challenging with LACV at a MOI of 5 PFU/cell. The cells were then analyzed for Gc expression at 8 hpi. As shown in Figure 10B, cells treated with media derived from LACV-infected HaCaT (gray bars) showed a dilution-dependent reduction in Gc expression compared to cells treated with media from mock-infected cells (black bars). To identify potential cytokines produced by LACV-infected HaCaT cells, media was collected from mock-infected or LACV-infected cells at different hpi, treated with UV light, and analyzed by a Biolegend LegendPlex assay. As shown in Table 2, mock-infected cells produced basal levels of IFN-λ (both 1 and 2/3) and IFN-β, that did not change substantially over time. By contrast, media from LACV-infected cells contained increasing levels of all three cytokines, and levels peaked at 16 hpi. Other cytokines were assayed in the BioLegend LegendPlex such as Tumor Necrosis Factor Alpha (TNF-α) and interleukin-1 (IL-1) but levels of these cytokines were less than 2-fold higher as compared to media from mock-infected cells at 16 hpi. To determine if LACV infection induced IFN-λ1, IFN-λ2/3 and/or IFN-β at the mRNA level, total RNA was harvested from mock-infected or LACV-infected HaCaT cells and analyzed by qPCR. As shown in Figure 10C, mRNA expression peaked at 16 hpi for all three cytokines, with higher levels of mRNA for IFN-β as compared to IFN-λ.

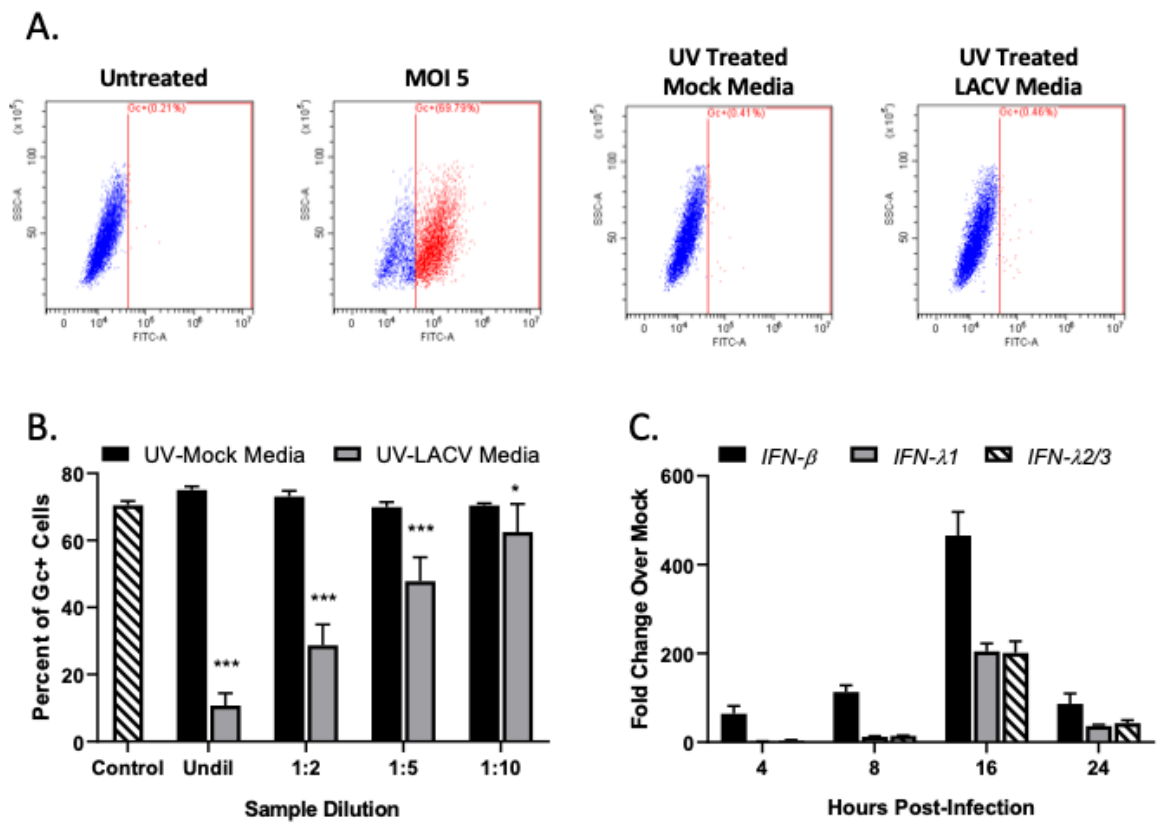


Figure 10: LACV-infected human keratinocytes express antiviral products.

HaCaT cells were mock- infected or infected with LACV at a MOI of 5 and media was collected at 8 hpi. (A) Media was treated with UV light to inactivate virus and then tested for loss of infectivity on naïve HaCaT cells. At 24 h post treatment, the percentage of infected cells was determined by Gc staining. Untreated and MOI 5 represent negative and positive controls for antibody staining. (B) HaCaT cells were treated for 16 h with the indicated dilutions of media from mock-infected (black bars) or LACV-infected cells (gray bars). Cells were then infected with LACV at a MOI of 5 and Gc staining was determined at 8 hpi. Untreated cells were also infected as a control (striped bar). (C) HaCaT Cells were infected at a MOI of 5, and total

cellular RNA was extracted at the indicated hpi and evaluated for IFN-β, IFN-λ and IFN-λ 2/3 expression by RT-qPCR.

Values are the mean of three replicates with error bars indicating standard deviation and * indicating p-values of <0.05 and *** indicating p-values of < 0.001.

Table 2: Cytokine levels expressed by LACV infected Keratinocytes as determined by BioLegend's LEGENDplexTM immunoassay

Cytokine	**Mock HaCaT (pg/ml)**			**Infected HaCaT (pg/ml)**		
Hours post Infection	**4 hr**	**8 hr**	**16 hr**	**4 hr**	**8 hr**	**16 hr**
IFN-λ1	14.9	13.8	14.0	13.4	584.9	1486.6
IFN-λ2/3	1.8	2.4	1.4	1.6	18.1	45.2
IFN-β	2.1	3.0	1.3	3.2	2.1	21.0

To determine if HaCaT cells can respond to IFNs and enter an antiviral state, naïve cells were treated with various concentrations of exogenous IFNs. Cells were then infected at a MOI of 5 PFU/cell with LACV and analyzed by flow cytometry for Gc expression. As seen in Figure 11A, pre-treatment with either type of IFN leads to a significant dose-dependent decrease in percent of Gc-expressing cells as compared to untreated cells. Infection of HaCaT cells was reduced to ~10% by 10 U/mL of IFN-β. In contrast, to reduce infection to the same level, a 100-fold higher concentration (1000 U/mL) of IFN-λ1 was required.

To determine if concentrations of IFNs released from infected HaCaT cells were sufficient to inhibit LACV infection, cells were treated with IFN levels detected by the LegendPlex assay shown in Table 2 and then challenged by LACV infection. IFN-β generated at an early time of 8 hpi (2.1 pg/mL) was sufficient to significantly inhibit LACV infection of

HaCaT cells, while levels detected at 16 hpi (21 pg/mL) reduced infection to <10%. By contrast, higher levels of IFN-λ1 generated at late times of 16 hpi (1486 pg/mL) were only partially able to inhibit LACV infection (Figure 11B). Importantly, reduction in infection by combined treatment of HaCaT with both cytokines was not significantly different from IFN-β treatment alone, indicating there is little synergistic effect with both IFN-λ1 and IFN-β (Figure 11B). Type I and Type III IFNs signal through the JAK/STAT pathway to induce an antiviral state. To directly test the role of IFNs in restricting LACV spread, HaCaT cells were treated with 1 μM of the JAK-1/2 inhibitor Ruxolitinib and subsequently infected at a MOI of 0.5 PFU/cell. This MOI was chosen to ensure a significant number of infected (~30–50%) and non-infected cells at early timepoints post-infection. At timepoints shown in Figure 11C, cells were analyzed for Gc expression. At 24 hpi, there was a significant increase in the percentage of Gc-expressing cells after Ruxolitinib treatment (white bars) as compared to control treated cells (cross hatched bars). Since Ruxolitinib can inhibit both type I and type III IFN signaling, neutralizing antibodies were used to distinguish the roles of IFN-β and IFN-λ. HaCaT cells were infected at a MOI of 0.5 PFU/cell and were treated with neutralizing antibodies against IFN-β or against IFN-λ1 and IFN-λ2/3. At 24 hpi, cells were analyzed by flow cytometry for Gc expression. As seen in Figure 11D, there is a significant increase in the percentage of Gc-expression in cells treated with anti-IFN-β (white bar) which had ~50% expression as compared to ~30% in the isotype control (hatched bar). Cells treated with the IFN-λ antibodies also had a small but significant increase, from ~40% to 30%, in the percentage of infected cells as compared to the isotype control.

Taken together, these data show that HaCaT cells produce type I IFN-β and type III IFN-λ in response to LACV infection, which limits the spread of a LACV infection in a population of

HaCaT cells. The antiviral response is primarily driven through IFN-β, with a minor contribution by IFN-λ.

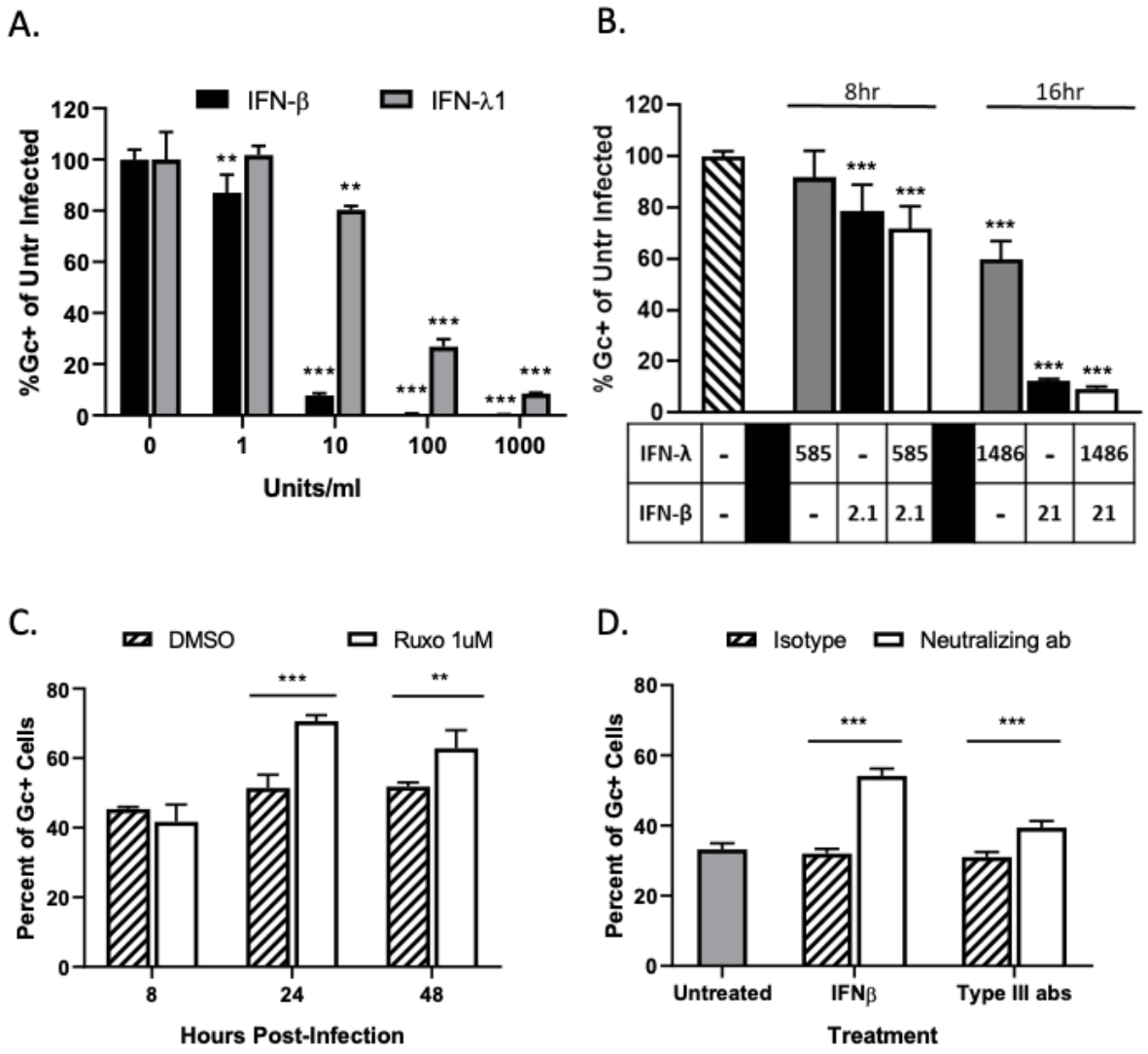


Figure 11:LACV spread through a population HaCaT cells is limited by type I and type III IFN responses.

LACV spread through a population of HaCaT cells is limited by type I and type III IFN responses. (A and B) HaCaT cells were mock-treated or treated for 16 h with the indicated

concentrations of exogenous IFN-β or IFN-λ1 (A), or a combination of both cytokines (B) before infection with LACV at a MOI of 5. At 8 hpi, cells were analyzed by flow cytometry for LACV Gc expression. Data is shown as percentage of untreated infected control. (C) HaCaT cells were treated with DMSO as a control or 1 μM Ruxolitinib for 16 hrs, followed by LACV infection at a MOI of 0.5. At indicated times, cells were analyzed by flow cytometry for Gc expression. (D) HaCat cells were first infected at a MOI of 0.5 PFU/cell and were then treated with 3 μg of neutralizing antibodies against IFN-β, a combination of antibodies to Type III IFNs (IL29, IL28a, and Human IFN Lambda Receptor 1) or the corresponding isotype control antibodies. At 24 hpi, cells were analyzed by flow cytometry for LACV Gc expression. In panel D, gray bar represents the percentage of Gc-positive cells in untreated LACV-infected cells. Values are the mean of three replicates with error bars indicating standard deviation and ** indicating p-values of <0.01 and *** indicating p-values of < 0.001.

LACV Infection Induces Cell Death in Non-Infected Bystander HaCaT Cells

As seen in the above data in Figure 9B, HaCaT cells infected at an intermediate MOI (0.5 PFU/cell) show that ~70% of the population stained positive for PI at 48 hpi, despite only showing ~40% cells being Gc-positive. These data raise the hypothesis that non-infected HaCaT cells in the population are undergoing cell death. To quantitate death of infected vs. non-infected cells, HaCaT cells were infected with LACV at a MOI of 0.5 PFU/cell and at different times post-infection; cells were simultaneously stained with a fixable cell viability dye (Zombie Red™) as well as anti-Gc antibody to quantitate virus-infected cells. In the analysis, Zombie Red was used to exclude non-viable cells and from this, the percentage of Gc was quantitated within the

viable population. As shown in Figure 12A, at 24 hpi there were approximately ~1500 viable cells that were Gc-positive and Gc-negative. However, by 72 hpi the number of viable cells was significantly reduced to ~250 Gc-positive cells and ~500 Gc-negative cells. This supports the hypothesis that during LACV infection of HaCaT cells, death occurs in both infected and non-infected cells.

A media-transfer experiment was used to test the role of extracellular factors in the observed bystander killing during LACV infection. HaCaT cells were mock-infected or infected with LACV at a high MOI and media was collected at 24 (denoted M24 and L24, respectively) and 48 hpi (M48 and L48). Media was exposed to UV light, and virus inactivation was confirmed as described above. Media was then used to treat naïve non-infected HaCaT cells for 48 h and cell viability was quantified by PI staining. As seen in Figure 12B, there is significant cell death in naïve cells treated with media from infected HaCaT cells (L24 and L48) versus those treated with media from mock-infected HaCaT (M24 and M48). Similar results were seen using an MTT proliferation assay. These data indicate that LACV-infected HaCaT cells produce factors that can induce cell death in naïve non-infected cells.

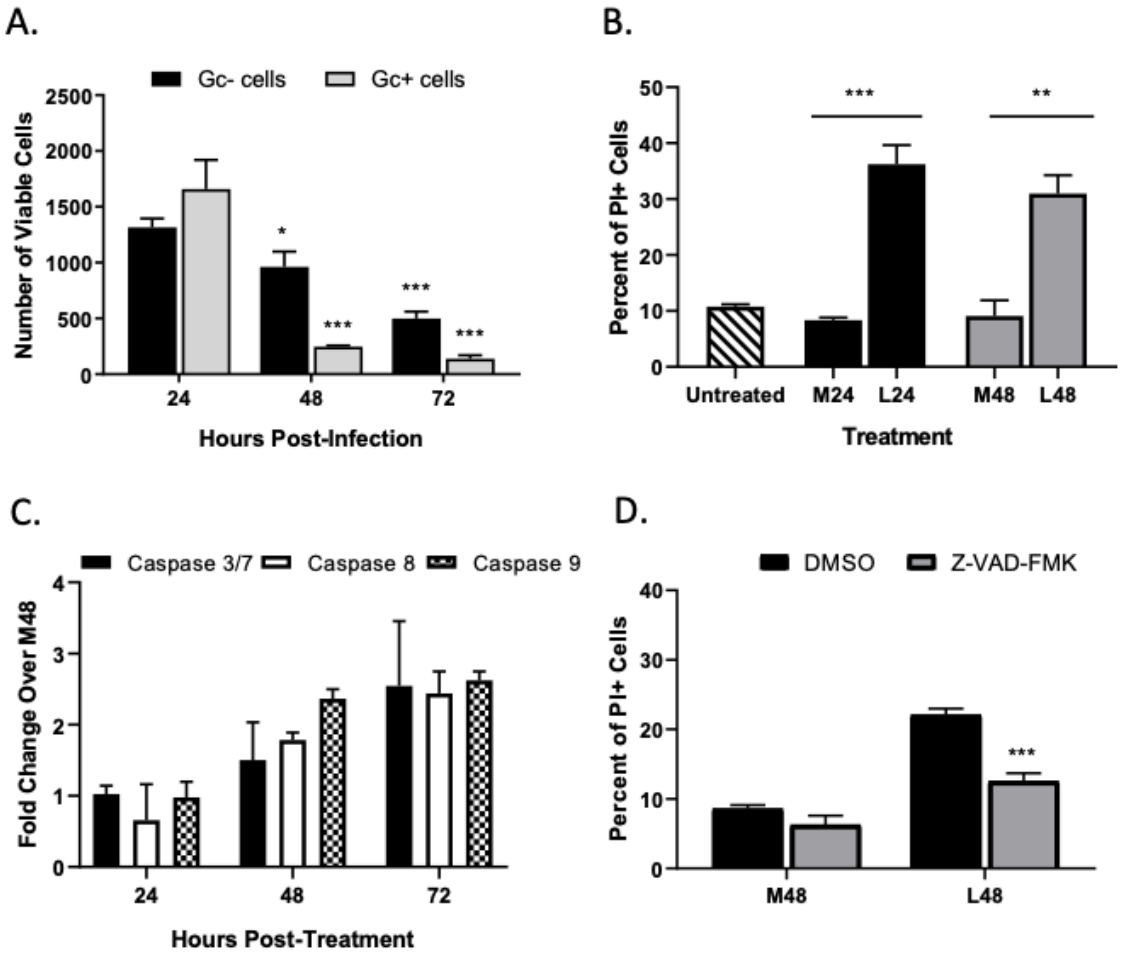


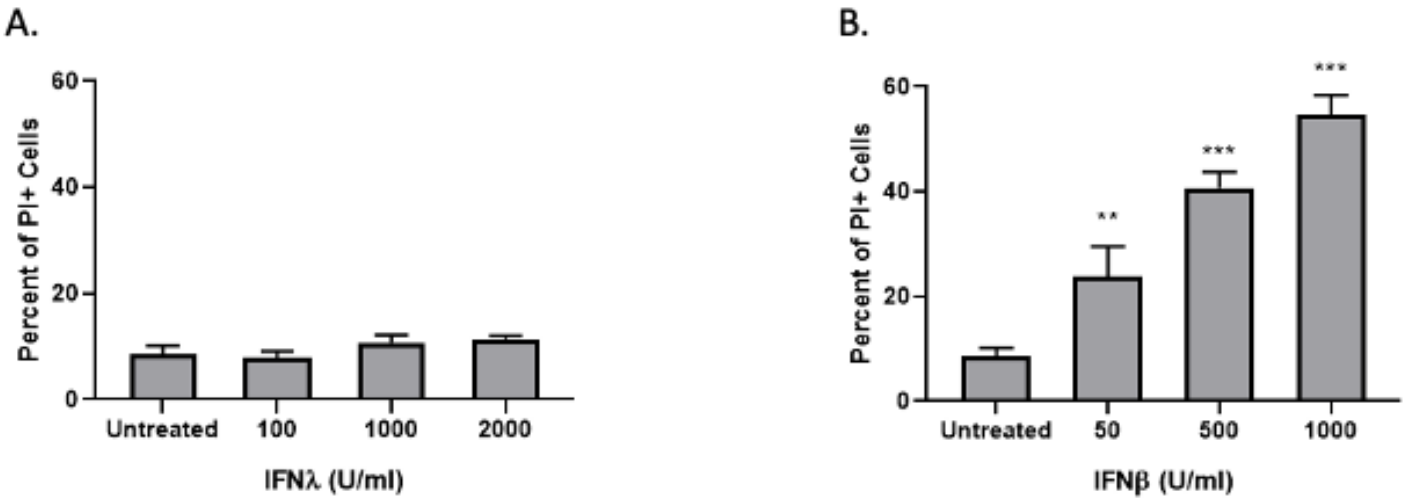
Figure 12: LACV infection of HaCaT cells induces cell death in non-infected bystander cells. LACV infection of HaCaT cells induces cell death in non-infected bystander cells. (A) HaCaT cells were infected at a MOI 0.5 PFU/cell. At the indicated hpi, cells were harvested and analyzed by flow cytometry for LACV Gc expression and for cell viability using Zombie Red™ dye. Data are expressed as number of Gc-positive and Gc-negative viable cells. (B–D) Media from LACV-infected or mock-infected HaCaT cells were collected at 24 hpi (B) and 48 hpi (B–D) and were UV-treated to inactivate virus. (B) Naïve cells were treated for 48 h with UV-inactivated media and cell viability was determined by PI staining. (C) Alternatively, cells were

treated with UV-inactivated media, and caspase activity was determined by Caspase-Glo-3/7, -9 or -8 assays. Data are expressed as fold change over that seen with cultures treated with media from mock-infected cells (M48). (D) Naïve HaCaT cells were pre-treated with 40 μM of Z-VAD-FMK followed by treatment with the indicated media from mock-infected (M48) or LACV-infected cells (L48). After treatment for 48 h, cell viability was determined by PI staining. Values are the mean of three replicates with error bars indicating standard deviation and * indicating p-values of <0.05, ** indicating p-values of <0.01 and *** indicating p-values of < 0.001.

To determine if caspases are activated during bystander cell death, HaCaT cells were treated with UV-inactivated media collected 48 hpi from LACV-infected HaCaT cells, and caspase activity was measured by Caspase-Glo assays. Increased caspase activation was not seen until 48 h post treatment of cells, and by 72 h post treatment there was a ~2–3-fold activation of both initiator caspase -8 and -9, and executioner caspases-3/7 as compared to cells treated with media from mock-infected cells (M48) (Figure 12C). To confirm that observed bystander cell death was caspase-dependent, HaCaT cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK followed by treatment with UV-inactivated media, and cell viability was quantitated by PI staining after 48 h (Figure 12D). While cell death was significantly reduced by Z-VAD-FMK, PI staining was not reduced to baseline staining induced by control media. These results show that bystander cell death can be induced by factors from LACV-infected HaCaT cells, and this death is due in part to caspase activation.

Bystander Cell Death Observed in Non-Infected HaCaT Cells is IFN-Dependent

We hypothesized that IFNs were responsible for the bystander cell death observed during LACV infection of HaCaT cells. To test this, naïve HaCaT cells were treated with different concentrations of exogenous IFN-λ1 or IFN-β for 48 h and then analyzed for PI staining. At all concentrations tested, IFN-λ1 did not induce any detectable increase in death (Figure 13A). By contrast, IFN-β induced dose-dependent increases in cell death, with 1000 U/mL treatment resulting ~45% PI staining in the population (Figure 13B). To confirm that IFN contributed to the bystander effect, naïve HaCaT cells were treated with UV-inactivated media in the presence of either the Jak 1/2 inhibitor Ruxolitinib or with neutralizing antibodies against IFN-β or all type III IFNs. At 48 h post treatment, cells were analyzed for PI staining. Cells that were treated with UV-inactivated media along with Ruxolitinib or along with IFN neutralizing antibodies had a significant decrease in PI staining as compared to treatment with the corresponding vehicle control or isotype control antibodies (Figure 13C). Together, these data indicate that HaCaT cells are susceptible to IFN-β-induced death, and that IFN-β contributes to bystander death of non-infected cells during multi-cycle LACV infection.



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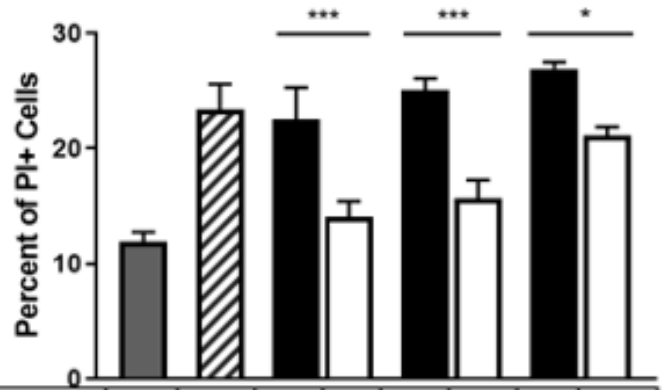

UV-Mock Media	+	0	0	0	0	0	0	0
UV-LACV Media	0	+	+	+	+	+	+	+
Ruxo	0	0	-	+	0	0	0	0
αIFN-β	0	0	0	0	-	+	0	0
αIFN Type III	0	0	0	0	0	0	-	+

Figure 13: LACV infection induces bystander HaCaT cell death mediated by type I and type III IFN.

(A and B) Naïve HaCaT cells were treated with indicated concentrations of IFN-λ (A) or IFN-β (B), and cell viability was determined 48 h later by PI staining. (C) Media collected at 48 hpi from LACV- infected or mock-infected HaCaT cells was UV-treated to inactivate virus. Naïve cells were pretreated with 1 μM Ruxolitinib or with 3 μg of neutralizing antibodies against IFN-β, or a combination of antibodies that block IL29, IL28a, and Human IFN Lambda Receptor 1 (white bars). Corresponding isotype controls or DMSO controls were also included (black bars). Symbols on the table denote + as treatment, − as corresponding control, and 0 as no treatment. After 48 h of treatment with M48 or L48 media, cell viability was determined by PI staining. Controls included cells treated with UV-inactivated media from mock-infected cells (gray bar) or from LACV-infected cells (striped bar). Values are the mean of three replicates with error bars indicating standard deviation and * indicating *p*-values of <0.05, and *** indicating *p*-values of < 0.001

Spread of LACV Infection Is Restricted in Dermal Fibroblasts without Bystander Non-Infected Cell Death

We extended our studies to another skin cell type that is relevant to LACV infections - dermal fibroblasts. The dermal fibroblast cell line Hs27 was infected with LACV at three different MOIs - 5, 0.5 and 0.05. At the indicated hpi, cells were analyzed by flow cytometry for Gc expression (Figure 14A), or in a parallel experiment were stained with PI to quantitate cell viability (Figure 14B). Similar to the results described above for HaCaT keratinocyte cells, LACV could productively infect Hs27 cells at high MOI, and multi-cycle spread of the infection was restricted in Hs27 fibroblasts. This is evident by the finding of fewer Gc-positive Hs27 cells

over time after infection at MOIs of 0.5 and 0.05 (Figure 14A). However, unlike HaCaT cells, cell death in LACV infected Hs27 cells was delayed at all times post infection and with all MOI tested (Figure 14B).

To directly test for bystander cell killing, Hs27 cells were infected at an MOI of 0.5 and at different hpi, cells were simultaneously stained with a fixable cell viability dye (Zombie Red) as well as anti-Gc antibody to quantitate Gc expression (Figure 14C). Interestingly, while viability in Gc-positive cells (stippled bars) significantly decreased over time, viability of Gc-negative Hs27 cells (black bars) was not altered over time. These results in fibroblast Hs27 cells contrast with the loss of viability of both Gc-positive and Gc-negative keratinocyte HaCaT cells, and suggest that bystander cell death in non-infected cells differs between Hs27 fibroblast and HaCaT keratinocytes.

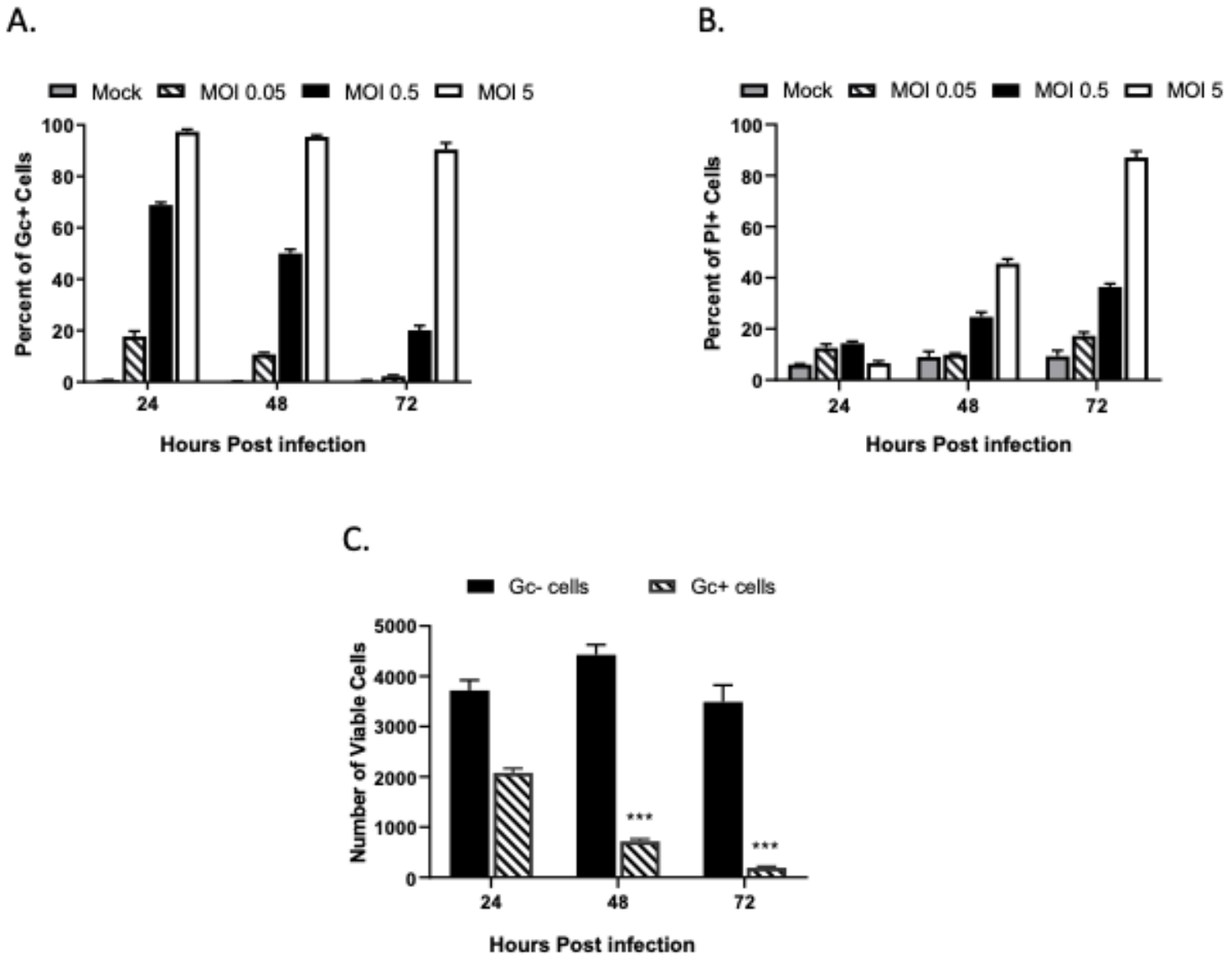

Figure 14: LACV infection of human dermal fibroblasts does not induce bystander cell death.

(A-B) Hs27 dermal fibroblast cells were mock infected or LACV infected at an MOI of 0.05, 0.5 or 5. At the indicated hpi, cells were analyzed by flow cytometry for LACV Gc expression (A). In parallel, cell viability was determined by PI staining (B). Values are the mean of three replicates with error bars indicating standard deviation. (C) Hs27 cells were infected at an MOI 0.5. At indicated hpi, cells were harvested, stained and analyzed by flow cytometry for LACV Gc expression and for cell viability using Zombie Red™ fixable dye. Data are expressed as the number of Gc-positive and Gc-negative cells that are viable. Values are the mean of three replicates with error bars indicating standard deviation and *** indicating p-values of < 0.001.

CHAPTER 5: DISCUSSION

Host innate immune interactions at the initial site of entry of arboviruses have the potential to limit or to enhance virus replication. Due to the nature of arbovirus entry, we hypothesize that interactions between an arbovirus and blood represents one of the first interfaces between an arbovirus and innate immune factors. We examined the interactions of human serum with insect cell- and human-cell derived LACV. Our results show that factors in serum do not neutralize, but instead can enhance insect cell-derived LACV infectivity. The observed enhancement occurs at early steps of replication and is partly dependent on antibodies. Strikingly, serum had no observable effect on human cell-derived LACV.

Following virus delivery by a mosquito, LACV must enter a host cell to begin replication. To mimic this second phase of replication in a human host, we have examined the initial replication and spread of LACV through human keratinocytes in culture. Our work was initiated by the need to understand the potential for these cells to expand or limit virus replication. HaCaT keratinocytes are highly susceptible to LACV infection at a high MOI, producing high levels of viral proteins and infectious particles as well as inducing extensive cell killing. Unexpectedly however, multicycle replication following low MOI LACV infection is restricted in keratinocytes cultures. Keratinocytes limit spread of LACV infection by mounting a culture-wide antiviral response through the production of both type I and type III IFN. Our most striking finding was that during multicycle LACV replication, neighboring non-infected keratinocytes were also killed through IFN-β-dependent and caspase-dependent mechanisms.

Based on these findings, we propose a model whereby LACV is delivered by a mosquito into host skin where serum factors facilitate virus entry into skin resident cells such as

keratinocytes. As a result of LACV infection, keratinocytes mount an antiviral response through both induction of an antiviral state as well as killing potential new target cells which could support further centers of replication to limit LACV spread. The combined outcomes of initial host defense mechanisms can then dictate the success of virus infection.

LACV is Resistant to Complement-Mediated Neutralization

Serum factors can include a variety of molecules with potential broad antiviral activity, one of the most potent being complement. The complement system is comprised of more than 40 proteins that undergo a proteolytic cleavage cascade upon pathogen recognition, resulting in direct lysis of a pathogen due to the formation of a membrane attack complex or, opsonization by proteolytic fragments for enhanced uptake by phagocytes (Blue et al., 2004; Stoermer and Morrison, 2011). Surprisingly, we found that insect cell-derived LACV and HaCaT-LACV were completely resistant to complement-mediated neutralization *in vitro*. Since bunyaviruses have small genomes and limited number of encoded proteins, it is likely that this complement resistance involves high-jacking of host inhibitor proteins similar to what we have shown for other enveloped RNA viruses (Johnson et al., 2009, 2012; Li and Parks, 2018). Future studies will attempt to characterize LACV complement evasion mechanisms.

Serum Enhances Insect-Derived LACV Infectivity

Our immune-staining data indicate that serum-mediated enhancement of I-LACV infectivity occurs at an early step in replication. This suggests that serum could act on I-LACV during attachment, internalization, or membrane fusion. Possible mechanisms of action include:

1) increased attachment to cells via utilization of an alternate receptor. For example, Ebola virus (EBOV) binds antibodies and the complement factor C1q to enhance attachment to cells independent of its cognate receptor (Takada, Feldmann et al., 2003); 2) Altering internalization mechanisms to facilitate virus entry as seen in the enhancement of EBOV infectivity by Ficolin-1 which is in part due to changes in micropinocytosis, the preferred mechanism of entry for EBOV (Favier et al., 2016); 3) Enhancing viral glycoprotein fusion with target cells as shown by Haslwanter et al. (2017) in Tick-borne encephalitis where antibody binding leads to conformational changes of the fusion protein that allow for enhanced pH independent fusion. Identifying factors involved in LACV enhancement will allow us to identify what step in viral replication is altered by LACV-serum interactions.

Using commercial serum that is depleted of antibodies, we showed that removal of antibodies decreased the ability of serum to enhance I-LACV infectivity. It is unlikely that this enhancement is due to antigen specific antibodies resulting from prior infection with LACV. This is because all human donors tested here show some degree of enhancement. In addition, we show that enhancement of I-LACV infectivity is seen with serum from multiple species, which included animals within and outside of the normal LACV host animal tropism. We hypothesize that antibodies involved in enhancement are not LACV-specific, but rather are natural polyreactive antibodies (nAbs). These antibodies are present in serum before exposure to any pathogens and thus, are considered part of innate immunity (Holodick et al., 2017; Palma et al., 2018). They are known for their broad activity against self-antigens generated in normal cell processes such as oxidation and apoptosis, however they also provide protection against viral, bacterial, fungal and protozoan pathogens (Boes et al., 1998; Holodick et al., 2017; Ochsenbein et al.,1999;

Subramaniam et al., 2010). In antibody depletion studies, we showed that enhancement of LACV infection was significantly reduced by the depletion of IgG antibodies, but it was not reduced to the same levels as control samples of I-LACV alone. As described below, there are two possible mechanisms for enhancement of I-LACV infectivity.

Antibody dependent-enhancement (ADE) has been extensively characterized in the case of Dengue virus, Zika virus, and HIV (Dejnirattisai et al., 2016; Katzelnick et al., 2017; Takada and Kawaoka, 2003; Szabo et al., 1999). Traditional ADE has been observed only in immune cells displaying Fcγ receptors specific for IgG antibodies. All our studies assayed serum enhancement on Vero cells, an indicator cell line of endothelial origin, that has no known Fc receptors (Rodrigo et al., 2009; Taylor A. et al., 2015). This suggests that enhancement of LACV infectivity does not require Fc receptors, and thus it is unlikely that this is mediated by traditional ADE. If antibodies are involved in LACV enhancement as suggested by our depletion and sucrose sedimentation assays, then we hypothesize that they require other soluble factors that would mediate the attachment to the host cell such as lectins which have been shown to interact with natural IgG antibodies (Panda et al., 2013, 2014).

Alternatively, previous studies that have shown that depletion of serum components in commercial preparations can unexpectedly also deplete other factors (Brady et al., 2014). Thus, it is possible that antibodies *per se* are not involved in I-LACV enhancement, but rather reflect loss of an antibody-associated factor - possible factors include lectins, such as MBL and fictions, which can enhance Ebola and HIV infection (Favier et al., 2016; Brudner et al., 2013; Ouellet et al., 2005). Future studies will seek to address these models to identify the enhancement factor and mechanism of action.

Differential interactions of Insect- and Human- Derived LACV with Human Serum

Arbovirus transmission to humans is dependent upon successful infection, replication and dissemination in two different organisms – insect and mammal. Our study focused on insect cell-derived LACV as this form of virus is delivered as inoculum at the initial site of entry in the skin following an insect bite. Virus is then produced from subsequent replication in mammalian cells. To mimic this second phase of replication in a human host, we carried out neutralization assays with keratinocyte-derived LACV. In agreement with our results with I-LACV, HaCaT-LACV was not neutralized by complement or antibodies in normal human serum. Unexpectedly and in contrast to I-lACV, HaCaT-LACV infectivity was stable *in vitro* when incubated alone and treatment with serum did not enhance infectivity. The striking differences in the two viruses derived from two different cell types may reflect differences in processing by cell machinery such as glycosylation or in lipid content of cellular membranes. Mammalian cells generally have a high cholesterol content which has been shown to enhance packing of acyl chains of phospholipids in the bilayer, increase mechanical strength and reduce permeability of the cell membrane (Xu et al., 2005; Hafer et al., 2009). In contrast, mosquitoes cannot synthesize cholesterol de novo and are considered to be cholesterol auxotrophs since they acquire cholesterol from their diets. Thus, insect cell membranes have significantly less cholesterol in their membranes compared to animal cells (Krebs and Lan, 2003; Xu et al., 2005). Studies conducted on Sindbis virus suggest that cholesterol content can affect virus stability. When Sindbis viruses were grown in low cholesterol environments they were more structurally fragile than viruses grown in cholesterol-rich environments (Hafer et al., 2009). Thus, it is possible that I-LACV vs HaCaT-LACV stability could be a result of lipid content in the respective cells.

Glycosylation of membrane proteins is also known to differ between insect and mammalian cells. Mosquito cell N-acetylglucosaminyl, -galactosyl, and sialyltransferases have negligible activities, but in contrast, are essential for assembly of N-glycans in mammalian glycoproteins (Butters et al., 1981). As opposed to complex N-glycosylation, mosquito cell glycoproteins exhibit high mannose and hybrid glycosylation (Rendic et al., 2008). Dengue virus has been shown to have differential patterns of membrane protein glycosylation between virus derived from insect and mammalian cells (Hacker et al., 2009). Insect cell-derived West Nile virus and Sindbis virus bind DC-SIGN, an attachment factor on dendritic cells, to higher levels than the respective mammalian cell-derived virus (Klimstra et al., 2003), reflecting high levels of mannose-containing structures on insect cell-derived glyoproteins which are required for DC-SIGN binding (Feinberg et al., 2001; Klimstra et al., 2003). LACV has extensive glycosylation in the Gn and Gc glycoproteins shown by studies carried out on BUNV, a prototype bunyavirus, which showed several N-glycosylation sites that are conserved across the *Orthobunyavirus* genus (Shi et al., 2005; Madoff & Lenard et al., 1982). Future studies will determine if glycosylation differences result in altered interactions with serum.

HaCaT Cell Line

The HaCaT keratinocyte cells used in this study are from a spontaneously transformed cell line that has been used extensively to study various skin cell processes as well as skin inflammatory conditions, including psoriasis, UV damage and, atopic dermatitis (Cinque et al., 2006; Gil et al., 2019; Kimura et al., 2012; Zampetti et al., 2009). HaCaT cells show normal differentiation patterns and can be induced to secrete various inflammatory cytokines in the same

manner as primary keratinocytes (Colombo et al., 2017; Deyrieux et al., 2007;). In this thesis work, HaCaT cells were maintained at confluencies less than 80% and low passage number for consistency of the culture and to prevent differentiation. Interestingly, we have noted that higher passage cells show phenotypes consistent with differentiation, and these cells show a lower susceptibility to infection with LACV and in their rate and extent of virus-induced death. The role of differentiation of skin cells and arbovirus infection will be an interesting area for future studies.

Human Keratinocytes Produce Cytokines that Limit Spread of LACV

IFNs have been extensively studied in the context of viral infections as factors that limit virus spread by paracrine and autocrine signaling through a population of cells (Kotenko et al., 2003; Levy et al., 2001; Samuel et al., 2001). In the current study we show that LACV infection of HaCaT cells results in production of both IFN-β and IFN-λ as early as 8 hrs after infection, and this correlates with detection of high levels of viral N and Gc proteins. Prior work has shown that the LACV nonstructural protein NSs can prevent type I IFN induction in some cell types by degradation of the RBP1 subunit of RNA polymerase II (Verbruggen et al., 2011). Conversely however, others have reported production of IFN-β in the presence of LACV NSs in cell types such as myeloid dendritic cells and microglial cells (Kallfass et al., 2012; Taylor et al., 2014). Thus, the ability of LACV to prevent induction of antiviral cytokines is very likely a function of differing cell types (e.g., epithelial vs. neuronal vs. keratinocyte) and/or cell species (mouse vs. human) used in a particular study. It is also noteworthy that our time course experiments show that IFN-λ was secreted by LACV infected HaCaT cells at much higher levels than IFN-β,

despite the presence of lower levels of IFN-λ mRNA compared to IFN-β mRNA. These data suggest that the pathways for production of extracellular type I and III IFNs in infected keratinocytes may have major differences, including their regulation at the level of translation or transport, as well as sensitivity to shutoff by viral antagonists (e.g., NSs protein) and cytopathic effects.

Despite HaCaT cells producing lower levels of IFN-β compared to IFN-λ (e.g., Figure 4D), our antibody neutralization data show that restricted LACV replication was relieved by culturing with the JAK-1/2 inhibitor Ruxolitinib or with a neutralizing antibody to IFN-β. By contrast, IFN-λ pathways did not appear to play a major role in restriction of multi-cycle LACV replication. Similarly, in the absence of virus, naïve HaCaT cells entered an antiviral state capable of blocking LACV infection with much lower levels of IFN-β compared to IFN-λ. It is reported that IFN-β generates a more inflammatory and potent response than IFN-λ (Meager et al., 2005), which could be due to differences in receptor-ligand affinity, number of IFN receptors, or subsequent downstream signaling events. It is also possible that IFN-λ activates a different landscape of ISGs which are less effective in inhibiting LACV replication compared to those induced by IFN-β (Lazear, et al., 2019; Meager, et al., 2005). Interestingly, inhibition of these two IFN pathways (through Ruxolitinib and with combined neutralizing antibodies) was not sufficient to yield complete un-restricted virus replication throughout the entire population of keratinocyte cells, suggesting that alternative mechanisms besides IFN are in place to restrict multicycle LACV replication.

LACV Infection Induces Cell Death of Infected and Bystander Non-Infected Cells

High MOI LACV infection was very cytopathic to human keratinocyte cells, as evidenced by the visual cell rounding, cytotoxic release of cell materials and loss of intact cell membranes (Figure 2A-D). Our data on timing of cell killing and IFN induction after high MOI LACV infection are consistent with previous reports of LACV-induced killing of mouse neuronal cells through common RIG-I and MAVS pathways that activate both cytokine production as well as apoptosis (Mukherjee et al., 2013). In addition, others have shown that LACV NSs bears some sequence and functional similarities to drosophilia Reaper, a protein that induces apoptosis by translational shutoff and cytochrome C release (Colon-Ramos et al., 2003), suggesting a possible role in mimicking a cellular inducer of death. Keratinocyte cell killing by LACV was dependent on functions of caspase pathways, but the death pathways are likely to be complex and overlapping since both caspase-8 and -9 were induced simultaneously

Our most striking finding emerged from results that multicycle LACV infections at low MOIs led to higher amounts of keratinocyte cell death than could be accounted for by the percent of infected cells. Our dual staining of LACV-infected populations as well as media-transfer experiments showed 1) non-infected cells within the HaCaT population were dying, 2) that this death was similar to LACV infected cells by being at least in part caspase-dependent, and 3) that death was induced by extracellular factors secreted from the LACV-infected HaCaT cell population. Remarkably, naïve HaCaT cells were killed by treatment with IFN-β alone but not by IFN-λ treatment, and inhibition of IFN signaling with Ruxolitinib and with IFN-β neutralizing antibody reduced killing by media from LACV infected cells. Our experiments suggest that at early times after infection, IFN-β exerts a protective role in the population which

prevents further spread of the infection, but at later times post infection continuous IFN-β signaling has a pro-apoptotic effect on non-infected neighboring cells. Other work has shown that extended IFN signaling can lead to differential regulation of STAT protein expression and this can play a role in cell survival versus cell death (Meager et al., 2005). Several studies have shown that type I IFNs can induce cell death in various types of cells through different pathways. For example, IFN-β treatment led to death of neuroblastoma cells through activation of the OAS/RNAseL system, activation of PKR, and differential regulation of STATs as well as inhibition of pro-survival signaling by the PI3k/AKT pathway (Castelli et al., 1997; Dedoni et al.,2010). IFN-β induced cell death has also been studied in the context of cancer therapies. For example, IFN-β-mediated death of human cervical carcinoma cells can be through changes in the caspase-8/cFLIP balance in the death-inducing signaling complex (DISC) (Apelbaum et al., 2013), or through activation of NF-kb and subsequent expression of pro-apoptotic proteins such as Fas, Bax, and p53 (Dedoni et al., 2010). It is important to note that under the conditions of our experiments, death of LACV infected cells may be driven by both extracellular IFN-β autocrine signaling and by intracellular responses to LACV replication. Likewise, we have shown that death of non-infected bystander cells is driven by IFN-β, but it may also be influenced by the presence of other cellular or viral components (e.g., UV inactivated virions or proteins) that are released from infected cells. Future studies on IFN-β -mediated cell death pathways will elucidate the role that each play in the bystander cell death effect observed in LACV infections.

Similar to keratinocytes, human fibroblast cells were highly susceptible to LACV infection, and were restricted for multicycle spread through the cell population, but properties of the fibroblast infection differs from that of keratinocytes in several aspects. First, fibroblast cells

were more susceptible to infection at low MOIs compared to keratinocytes. Secondly, LACV-infected fibroblast cells showed much slower death rates compared to keratinocytes – at 48 hpi at high MOI, 40% of fibroblasts were PI+ compared to >80% for keratinocytes. Most strikingly, fibroblast cells did not show a clear bystander effect in which neighboring non-infected cells were killed during low MOI infection – cell death was only seen in those cells harboring LACV antigens and not in non-infected co-cultured cells. Thus, our data suggest that these two cell types are primed to respond differently to components produced by LACV infection.

Implications for Future Research

Our work on LACV interactions with human serum highlights the importance of host cell tropism in arbovirus studies. As described in the introduction, arboviruses have a dual life cycle consisting of insect-cell derived viruses which are initially delivered to animals by the mosquito via a blood meal, and mammalian cell-derived viruses which are the outcome of subsequent rounds of replication in the host. We have shown that insect cell grown LACV is less stable than mammalian grown LACV. Because mosquitoes are cold blooded organisms, it is likely that LACV within the salivary glands is maintained at significantly colder temperatures than in a mammalian host. The relative stability of insect-derived LACV then could be a reflection of the local environment: at temperatures below 37°C the virus is more stable and when transmitted into a mammalian host it makes use of serum factors to retain stability at higher temperatures until it can infect and begin initial replication. Alternatively, we observed significantly lower titers of virus growth in human cells vs insect cells. A more fragile virion could be a tradeoff to ensure higher levels of virus replication in insects, and factors within a mosquito such as salivary gland factors

could mimic the effects observed with serum and maintain stability of the virus. This would suggest then, that stability is not essential for survival of insect-derived virus. Although stability might not be essential for virus transmission it does present a potential target for therapeutics. If the arthropod-skin interface represents a step where virus structure is unstable, then topical prophylactic treatments that disrupt the virus envelope can be attractive options to prevent LACV infection.

Our work demonstrates that as a result of LACV cell source, LACV can elicit different a response in host immune defenses, consequently, it is important to carefully choose a cell virus source that is relevant to the topic of study. This raises the concern that results of studies that focus on virus-host interactions and use viruses grown in generic cell lines, could not be a true portrayal of in vivo host responses. In order to gain meaningful results, care must be taken to choose an adequate cell type that represents the in vivo virus cycle. For our studies, we chose to use insect cell-derived viruses, as our focus was on initial interactions upon virus delivery. Future studies will also study interactions with human cell-derived LACV in order to understand dissemination of the virus within the host. Our findings of differential interactions with serum also have implications for potential new antiviral approaches such as vaccine development as it highlights the importance of identifying epitopes to elicit an immune response that are present on the virus as it disseminates in the host. In addition to viral proteins that can elicit an immune response, structural differences in viruses grown in insect cells such as, envelope cholesterol content, could enhance the host immune response. As a result, using viruses grown in insect cells as vaccine candidates could yield a more effective vaccine than using mammalian cell-derived viruses which have acquired host cell components that would not be recognized by host immunity. Future

research will focus on characterizing the differences in insect cell-derived and human cell-derived LACV through proteomic and lipidomic studies to determine their impact on virus stability and its ability to infect a host.

In our study of LACV infection on human keratinocytes, we propose that LACV is introduced by insect bite into the skin tissue, where it can initially infect keratinocytes. As a result, keratinocytes mount an innate immune response that places nearby cells in an antiviral state and also induces cell death in the nearby non-infected cells as a mechanism to prevent further spread of LACV to new cells. An alternative model is that dermal fibroblasts serve as a site for initial virus replication. As a result of infection, fibroblast cells have been shown to be potent IFN-β producers and these IFNs can act in a paracrine manner on nearby cell populations such as keratinocytes. As a result of IFN I signaling then keratinocytes would be placed in an antiviral state and extended signaling would lead to induction of cell death in order to prevent spread of the virus. This study was carried out using cell cultures and as such the ability to study single cell responses is inadequate. We were unable to study infected vs uninfected cells within one population and as such, there are cell-by-cell specific responses that we could have missed. For instance, it is possible that uninfected cell death was dependent on cell cycle phase or on differentiation state of the cell, or that direct cell-to-cell contact might be required to induce cell death of uninfected cells. These are hypothesis that cannot be studied by observing a whole cell population which was the experimental basis of our study. Alternative approaches will use single cell analyses to better determine cell responses to infection. Single cell analysis such as microfluidics platforms would allow us to correlate perturbations (e.g. virus infection) to single cells to changes in the transcriptome or proteome, and this would allow us to examine cell-to-cell

interactions for example, how infected cells could influence uninfected cells (Shin et al., 2011; Yamanaka et al., 2012).

Disease susceptibility to LACV is age dependent and Taylor et al (2014) have shown that IFN I responses in myeloid dendritic cells are key determinants of pathogenesis. We have shown that IFN Type I plays a key role in restricting spread in a keratinocyte population and future studies will attempt to elucidate the role of keratinocyte IFN response in overall host infection and age-dependent disease susceptibility to LACV. This project indicates that understanding the initial interactions of LACV with host defense mechanisms is key to understanding determinates of host susceptibility to LACV infection and disease. Our study contributes to the understanding of these interactions, despite the limitations of in vitro assays, and testing of these interactions in isolation from other immune mechanisms. Future studies will attempt to dissect the combined action of innate immunity at the site of entry by using 3D skin models as well as animal models in order to determine how innate immunity contributes or limits the spread of LACV infection in a host.

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